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(54) Title: A SYSTEM FOR CELL-BASED SCREENING

(57) Abstract: The present invention provides methods, computer readable storage medium, and kits for cell state identification in cells, where the method includes providing arrays of cells that possess luminescently labelled cell identification and cell state reporter molecules that have distinguishable luminescent emission spectra; imaging the cells to obtain luminescent signals from the cell identification and the cell state reporter molecules; converting the luminescent signals into digital data to create a mask of the cell identification reporter molecule and the cell state reporter molecules; and determining the intensity of the cell state reporter molecule mask that co-localizes with the cell identification reporter molecule mask to identify the cell as being in a particular physiological state.

A SYSTEM FOR CELL-BASED SCREENING

(Case No. 97,022-Q)

Cross Reference

This application claims priority to U.S. Applications for Patent S/N 60/164,353, filed on November 9, 1999; and 60/176,504 filed January 18, 2000; and is related to co-pending U.S. applications 09/598,347 filed June 21, 2000; 09/352,171 filed July 12, 1999, 09/031,271 filed February 27, 1998; 09/293,209 filed April 16, 1999; 09/293,210 filed April 16, 1999; 09/398,965 filed September 17, 1999; 09/430,656 filed October 29, 1999; 09/513,783 filed February 24, 2000; 09/569,508 filed May 12, 2000; 09/632,552, filed August 4, 2000; 09/632,544, filed August 4, 2000; 09/650,937 filed August 29, 2000; and 09/676,217 filed September 29, 2000.

Field of The Invention

This invention is in the field of fluorescence-based cell and molecular biochemical assays for drug discovery, specifically for cell state identification.

Background of the Invention

Drug discovery, as currently practiced in the art, is a long, multiple step process involving identification of specific disease targets, development of an assay based on a specific target, validation of the assay, optimization and automation of the assay to produce a screen, high throughput screening of compound libraries using the assay to identify "hits", hit validation and hit compound optimization. The output of this process is a lead compound that goes into pre-clinical and, if validated, eventually into clinical trials. In this process, the screening phase is distinct from the assay development phases, and involves testing compound efficacy in living biological systems.

Historically, drug discovery is a slow and costly process, spanning numerous years and consuming hundreds of millions of dollars per drug created. Developments in the areas of genomics and high throughput screening have resulted in increased capacity and efficiency in the areas of target identification and volume of compounds screened. Significant advances in automated DNA sequencing, PCR application, positional cloning, hybridization arrays, and bioinformatics have greatly increased the number of genes (and gene fragments) encoding

potential drug screening targets. However, the basic scheme for drug screening remains the same.

Validation of genomic targets as points for therapeutic intervention using the existing methods and protocols has become a bottleneck in the drug discovery process due to the slow, manual methods employed, such as *in vivo* functional models, functional analysis of recombinant proteins, and stable cell line expression of candidate genes. Primary DNA sequence data acquired through automated sequencing does not permit identification of gene function, but can provide information about common "motifs" and specific gene homology when compared to known sequence databases. Genomic methods such as subtraction hybridization and RADE (rapid amplification of differential expression) can be used to identify genes that are up or down regulated in a disease state model. However, identification and validation still proceed down the same pathway. Some proteomic methods use protein identification (global expression arrays, 2D electrophoresis, combinatorial libraries) in combination with reverse genetics to identify candidate genes of interest. Such putative "disease associated sequences" or DAS isolated as intact cDNA are a great advantage to these methods, but they are identified by the hundreds without providing any information regarding type, activity, and distribution of the encoded protein. Choosing a subset of DAS as drug screening targets is "random", and thus extremely inefficient, without functional data to provide a mechanistic link with disease. It is necessary, therefore, to provide new technologies to rapidly screen DAS to establish biological function, thereby improving target validation and candidate optimization in drug discovery.

There are three major avenues for improving early drug discovery productivity. First, there is a need for tools that provide increased information handling capability. Bioinformatics has blossomed with the rapid development of DNA sequencing systems and the evolution of the genomics database. Genomics is beginning to play a critical role in the identification of potential new targets. Proteomics has become indispensable in relating structure and function of protein targets in order to predict drug interactions. However, the next level of biological complexity is the cell. Therefore, there is a need to acquire, manage and search multi-dimensional information from cells. Secondly, there is a need for higher throughput tools. Automation is a key to improving productivity as has already been demonstrated in DNA sequencing and high throughput primary screening. The instant invention provides for automated systems that extract multiple parameter information from cells that meet the need for higher throughput tools. The instant invention also provides for

miniaturizing the methods, thereby allowing increased throughput, while decreasing the volumes of reagents and test compounds required in each assay.

Radioactivity has been the dominant read-out in early drug discovery assays. However, the need for more information, higher throughput and miniaturization has caused a shift towards using fluorescence detection. Fluorescence-based reagents can yield more powerful, multiple parameter assays that are higher in throughput and information content and require lower volumes of reagents and test compounds. Fluorescence is also safer and less expensive than radioactivity-based methods.

Screening of cells treated with dyes and fluorescent reagents is well known in the art. There is a considerable body of literature related to genetic engineering of cells to produce fluorescent proteins, such as modified green fluorescent protein (GFP), as a reporter molecule.

Performing a screen on many thousands of compounds requires parallel handling and processing of many compounds and assay component reagents. Standard high throughput screens ("HTS") use mixtures of compounds and biological reagents along with some indicator compound loaded into arrays of wells in standard microtiter plates with 96 or 384 wells. The signal measured from each well, either fluorescence emission, optical density, or radioactivity, integrates the signal from all the material in the well giving an overall population average of all the molecules in the well.

In contrast to high throughput screens, various high-content screens ("HCS") have been developed to address the need for more detailed information about the temporal-spatial dynamics of cell constituents and processes. High-content screens automate the extraction of multicolor fluorescence information derived from specific fluorescence-based reagents incorporated into cells (Giuliano and Taylor (1995), *Curr. Op. Cell Biol.* 7:4; Giuliano et al. (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:405). Cells are analyzed using an optical system that can measure spatial, as well as temporal dynamics. (Farkas et al. (1993) *Ann. Rev. Physiol.* 55:785; Giuliano et al. (1990) In *Optical Microscopy for Biology*. B. Herman and K. Jacobson (eds.), pp. 543-557. Wiley-Liss, New York; Hahn et al (1992) *Nature* 359:736; Waggoner et al. (1996) *Hum. Pathol.* 27:494). The concept is to treat each cell as a "well" that has spatial and temporal information on the activities of the labeled constituents.

The types of biochemical and molecular information now accessible through fluorescence-based reagents applied to cells include ion concentrations, membrane potential, specific translocations, enzyme activities, gene expression, as well as the presence, amounts and patterns of metabolites, proteins, lipids, carbohydrates, and nucleic acid sequences

(DeBiasio et al., (1996) *Mol. Biol. Cell.* 7:1259;Giuliano et al., (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:405; Heim and Tsien, (1996) *Curr. Biol.* 6:178).

High-content screens can be performed on either fixed cells, using fluorescently labeled antibodies, biological ligands, and/or nucleic acid hybridization probes, or live cells using multicolor fluorescent indicators and "biosensors." The choice of fixed or live cell screens depends on the specific cell-based assay required.

Fixed cell assays are the simplest, since an array of initially living cells in a microtiter plate format can be treated with various compounds and doses being tested, then the cells can be fixed, labeled with specific reagents, and measured. No environmental control of the cells is required after fixation. Spatial information is acquired, but only at one time point. The availability of thousands of antibodies, ligands and nucleic acid hybridization probes that can be applied to cells makes this an attractive approach for many types of cell-based screens. The fixation and labeling steps can be automated, allowing efficient processing of assays.

Live cell assays are more sophisticated and powerful, since an array of living cells containing the desired reagents can be screened over time, as well as space. Environmental control of the cells (temperature, humidity, and carbon dioxide) is required during measurement, since the physiological health of the cells must be maintained for multiple fluorescence measurements over time. There is a growing list of fluorescent physiological indicators and "biosensors" that can report changes in biochemical and molecular activities within cells (Giuliano et al., (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:405; Hahn et al., (1993) In *Fluorescent and Luminescent Probes for Biological Activity*. W.T. Mason, (ed.), pp. 349-359, Academic Press, San Diego).

The availability and use of fluorescence-based reagents has helped to advance the development of both fixed and live cell high-content screens. Advances in instrumentation to automatically extract multicolor, high-content information has recently made it possible to develop HCS into an automated tool. An article by Taylor, et al. (*American Scientist* 80 (1992), p. 322-335) describes many of these methods and their applications. For example, Proffitt et. al. (*Cytometry* 24: 204-213 (1996)) describe a semi-automated fluorescence digital imaging system for quantifying relative cell numbers in situ in a variety of tissue culture plate formats, especially 96-well microtiter plates.

Scanning confocal microscope imaging (Go et al., (1997) *Analytical Biochemistry* 247:210-215; Goldman et al., (1995) *Experimental Cell Research* 221:311-319) and multiphoton microscope imaging (Denk et al., (1990) *Science* 248:73; Gratton et al., (1994)

Proc. of the Microscopical Society of America, pp. 154-155) are also well established methods for acquiring high resolution images of microscopic samples. The principle advantage of these optical systems is the very shallow depth of focus, which allows features of limited axial extent to be resolved against the background. For example, it is possible to resolve internal cytoplasmic features of adherent cells from the features on the cell surface. Because scanning multiphoton imaging requires very short duration pulsed laser systems to achieve the high photon flux required, fluorescence lifetimes can also be measured in these systems (Lakowicz et al., (1992) *Anal. Biochem.* 202:316-330; Gerrittsen et al. (1997), *J. of Fluorescence* 7:11-15)), providing additional capability for different detection modes. Small, reliable and relatively inexpensive laser systems, such as laser diode pumped lasers, are now available to allow multiphoton confocal microscopy to be applied in a fairly routine fashion.

The instant invention provides systems, methods, and screens for high content screening (HCS), and combined high throughput (HT)-HC screening that significantly improve target validation and candidate optimization by combining many cell screening formats with fluorescence-based molecular reagents and computer-based feature extraction, data analysis, and automation, resulting in increased quantity and speed of data collection, shortened cycle times, and, ultimately, faster evaluation of promising drug candidates. The instant invention also provides for miniaturizing the methods, thereby allowing increased throughput, while decreasing the volumes of reagents and test compounds required in each assay.

SUMMARY OF THE INVENTION

The present invention provides methods, computer readable storage medium, and kits cell state identification in cells, comprising (a) providing an array of locations containing cells, wherein the cells possess a luminescently labeled cell identification reporter molecule and one or more luminescently labeled cell state reporter molecules, wherein emission from the cell identification and the cell state luminescently labeled reporter molecules are distinguishable; (b) imaging multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the cell identification and the cell state reporter molecules; (c) converting the luminescent signals into digital data; (d) using the digital data to create a mask of the cell identification reporter molecule and the cell state reporter molecule; and (e) determining the intensity of the cell state reporter molecule mask that co-localizes with the

cell identification reporter molecule mask, wherein if this intensity is above or below a certain threshold, the cell is characterized as being in a particular physiological state.

In various embodiments, the physiological state to be detected include cell viability, pathogenic infection of cells, receptor internalization, cell type, adipogenesis, and foam cell formation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a diagram of the components of the cell-based scanning system.

Figure 2 shows a schematic of the microscope subassembly.

Figure 3 shows the camera subassembly.

Figure 4 illustrates cell scanning system process.

Figure 5 illustrates a user interface showing major functions to guide the user.

Figure 6 is a block diagram of the two platform architecture of the Dual Mode System for Cell Based Screening in which one platform uses a telescope lens to read all wells of a microtiter plate and a second platform that uses a higher magnification lens to read individual cells in a well.

Figure 7 is a detail of an optical system for a single platform architecture of the Dual Mode System for Cell Based Screening that uses a moveable 'telescope' lens to read all wells of a microtiter plate and a moveable higher magnification lens to read individual cells in a well.

Figure 8 is an illustration of the fluid delivery system for acquiring kinetic data on the Cell Based Screening System.

Figure 9 is a flow chart of processing step for the cell-based scanning system.

Figure 10 A-J illustrates the strategy of the Nuclear Translocation Assay.

Figure 11 is a flow chart defining the processing steps in the Dual Mode System for Cell Based Screening combining high throughput and high content screening of microtiter plates.

Figure 12 is a flow chart defining the processing steps in the High Throughput mode of the System for Cell Based Screening.

Figure 13 is a flow chart defining the processing steps in the High Content mode of the System for Cell Based Screening.

Figure 14 is a flow chart defining the processing steps required for acquiring kinetic data in the High Content mode of the System for Cell Based Screening.

Figure 15 is a flow chart defining the processing steps performed within a well during the acquisition of kinetic data.

Figure 16 is an example of data from a known inhibitor of translocation.

Figure 17 is an example of data from a known stimulator of translocation.

Figure 18 illustrates data presentation on a graphical display.

Figure 19 is an illustration of the data from the High Throughput mode of the System for Cell Based Screening, an example of the data passed to the High Content mode, the data acquired in the high content mode, and the results of the analysis of that data.

Figure 20 shows the measurement of a drug-induced cytoplasm to nuclear translocation.

Figure 21 illustrates a graphical user interface of the measurement shown in Figure 20.

Figure 22 illustrates a graphical user interface of the measurement shown in Fig. 20.

Figure 23 is a graph representing the kinetic data obtained from the measurements depicted in Fig. 20.

Figure 24 details a high-content screen of drug-induced apoptosis.

Figure 25 is a graphical representation of data from validation runs of the PTHR internalization screen.

Figure 26 is a flow chart for signal processing.

Figure 27 is a flow chart for an autofocusing procedure to be used in signal processing.

Figure 28 is a flow chart for object processing procedure to be used in signal processing.

Figure 29 shows a representative display of a PC screen showing receptor internalization data displaying the spot count of individual wells.

Figure 30 shows a representative display of a PC screen showing receptor internalization data displayed on a field by field basis.

Figure 31 **(A)** Flowchart of image acquisition initialization phase; **(B)** Flowchart of image acquisition iteration phase.

Figure 32 is a flowchart of the Positive State Detection Threshold Computation.

Figure 33 is a flowchart of Neurite Outgrowth Alternative Quantification Method. **(A)** Neuronal Nuclei Identification; **(B)** Neurite Outgrowth Quantification.

Figure 34 is a flowchart of the Cell State Detection Method (Morphological Method).

Figure 35 is a flowchart of the Cell State Detection Method (Blob Analysis Method).

DETAILED DESCRIPTION OF THE INVENTION

All cited patents, patent applications and other references are hereby incorporated by reference in their entirety. As used herein, the following terms have the specified meaning:

Markers of cellular domains. Luminescent probes that have high affinity for specific cellular constituents including specific organelles or molecules. These probes can either be small luminescent molecules or fluorescently tagged macromolecules used as "labeling reagents", "environmental indicators", or "biosensors."

Labeling reagents. Labeling reagents include, but are not limited to, luminescently labeled macromolecules including fluorescent protein analogs and biosensors, luminescent macromolecular chimeras including those formed with the green fluorescent protein and mutants thereof, luminescently labeled primary or secondary antibodies that react with cellular antigens involved in a physiological response, luminescent stains, dyes, and other small molecules.

Markers of cellular translocations. Luminescently tagged macromolecules or organelles that move from one cell domain to another during some cellular process or physiological response. Translocation markers can either simply report location relative to the markers of cellular domains or they can also be "biosensors" that report some biochemical or molecular activity as well.

Biosensors. Macromolecules consisting of a biological functional domain and a luminescent probe or probes that report the environmental changes that occur either internally or on their surface. A class of luminescently labeled macromolecules designed to sense and report these changes have been termed "fluorescent-protein biosensors". The protein component of the biosensor provides a highly evolved molecular recognition moiety. A fluorescent molecule attached to the protein component in the proximity of an active site transduces environmental changes into fluorescence signals that are detected using a system with an appropriate temporal and spatial resolution such as the cell scanning system of the present invention. Because the modulation of native protein activity within the living cell is reversible, and because fluorescent-protein biosensors can be designed to sense reversible changes in protein activity, these biosensors are essentially reusable.

Disease associated sequences ("DAS"). This term refers to nucleic acid sequences identified by standard techniques, such as primary DNA sequence data, genomic methods such as subtraction hybridization and RADE, and proteomic methods in combination with reverse

genetics, as being of drug candidate compounds. The term does not mean that the sequence is only associated with a disease state.

High content screening (HCS) can be used to measure the effects of drugs on complex molecular events such as signal transduction pathways, as well as cell functions including, but not limited to, apoptosis, cell division, cell adhesion, locomotion, exocytosis, and cell-cell communication. Multicolor fluorescence permits multiple targets and cell processes to be assayed in a single screen. Cross-correlation of cellular responses will yield a wealth of information required for target validation and lead optimization.

In one aspect of the present invention, a cell screening system is provided comprising a high magnification fluorescence optical system having a microscope objective, an XY stage adapted for holding a plate with an array of locations for holding cells and having a means for moving the plate to align the locations with the microscope objective and a means for moving the plate in the direction to effect focusing; a digital camera; a light source having optical means for directing excitation light to cells in the array of locations and a means for directing fluorescent light emitted from the cells to the digital camera; and a computer means for receiving and processing digital data from the digital camera wherein the computer means includes: a digital frame grabber for receiving the images from the camera, a display for user interaction and display of assay results, digital storage media for data storage and archiving, and means for control, acquisition, processing and display of results.

Figure 1 is a schematic diagram of a preferred embodiment of the cell scanning system. An inverted fluorescence microscope is used 1, such as a Zeiss Axiovert inverted fluorescence microscope which uses standard objectives with magnification of 1-100x to the camera, and a white light source (e.g. 100W mercury-arc lamp or 75W xenon lamp) with power supply 2. There is an XY stage 3 to move the plate 4 in the XY direction over the microscope objective. A Z-axis focus drive 5 moves the objective in the Z direction for focusing. A joystick 6 provides for manual movement of the stage in the XYZ direction. A high resolution digital camera 7 acquires images from each well or location on the plate. There is a camera power supply 8, an automation controller 9 and a central processing unit 10. The PC 11 provides a display 12 and has associated software. The printer 13 provides for printing of a hard copy record.

Figure 2 is a schematic of one embodiment of the microscope assembly 1 of the invention, showing in more detail the XY stage 3, Z-axis focus drive 5, joystick 6, light source 2, and automation controller 9. Cables to the computer 15 and microscope 16,

respectively, are provided. In addition, Figure 2 shows a 96 well microtiter plate 17 which is moved on the XY stage 3 in the XY direction. Light from the light source 2 passes through the PC controlled shutter 18 to a motorized filter wheel 19 with excitation filters 20. The light passes into filter cube 25 which has a dichroic mirror 26 and an emission filter 27. Excitation light reflects off the dichroic mirror to the wells in the microtiter plate 17 and fluorescent light 28 passes through the dichroic mirror 26 and the emission filter 27 and to the digital camera 7.

Figure 3 shows a schematic drawing of a preferred camera assembly. The digital camera 7, which contains an automatic shutter for exposure control and a power supply 31, receives fluorescent light 28 from the microscope assembly. A digital cable 30 transports digital signals to the computer.

The standard optical configurations described above use microscope optics to directly produce an enlarged image of the specimen on the camera sensor in order to capture a high resolution image of the specimen. This optical system is commonly referred to as 'wide field' microscopy. Those skilled in the art of microscopy will recognize that a high resolution image of the specimen can be created by a variety of other optical systems, including, but not limited to, standard scanning confocal detection of a focused point or line of illumination scanned over the specimen (Go et al. 1997, *supra*), and multi-photon scanning confocal microscopy (Denk et al., 1990, *supra*), both of which can form images on a CCD detector or by synchronous digitization of the analog output of a photomultiplier tube.

In screening applications, it is often necessary to use a particular cell line, or primary cell culture, to take advantage of particular features of those cells. Those skilled in the art of cell culture will recognize that some cell lines are contact inhibited, meaning that they will stop growing when they become surrounded by other cells, while other cell lines will continue to grow under those conditions and the cells will literally pile up, forming many layers. An example of such a cell line is the HEK 293 (ATCC CRL-1573) line. An optical system that can acquire images of single cell layers in multilayer preparations is required for use with cell lines that tend to form layers. The large depth of field of wide field microscopes produces an image that is a projection through the many layers of cells, making analysis of subcellular spatial distributions extremely difficult in layer-forming cells. Alternatively, the very shallow depth of field that can be achieved on a confocal microscope, (about one micron), allows discrimination of a single cell layer at high resolution, simplifying the determination of the subcellular spatial distribution. Similarly, confocal imaging is preferable when detection modes such as fluorescence lifetime imaging are required.

The output of a standard confocal imaging attachment for a microscope is a digital image that can be converted to the same format as the images produced by the other cell screening system embodiments described above, and can therefore be processed in exactly the same way as those images. The overall control, acquisition and analysis in this embodiment is essentially the same. The optical configuration of the confocal microscope system, is essentially the same as that described above, except for the illuminator and detectors. Illumination and detection systems required for confocal microscopy have been designed as accessories to be attached to standard microscope optical systems such as that of the present invention (Zeiss, Germany). These alternative optical systems therefore can be easily integrated into the system as described above.

Figure 4 illustrates an alternative embodiment of the invention in which cell arrays are in microwells 40 on a microplate 41, described ion co-pending U.S. Application S/N 08/865,341, incorporated by reference herein in its entirety. Typically the microplate is 20 mm by 30 mm as compared to a standard 96 well microtiter plate which is 86 mm by 129 mm. The higher density array of cells on a microplate allows the microplate to be imaged at a low resolution of a few microns per pixel for high throughput and particular locations on the microplate to be imaged at a higher resolution of less than 0.5 microns per pixel. These two resolution modes help to improve the overall throughput of the system.

The microplate chamber 42 serves as a microfluidic delivery system for the addition of compounds to cells. The microplate 41 in the microplate chamber 42 is placed in an XY microplate reader 43. Digital data is processed as described above. The small size of this microplate system increases throughput, minimizes reagent volume and allows control of the distribution and placement of cells for fast and precise cell-based analysis. Processed data can be displayed on a PC screen 11 and made part of a bioinformatics data base 44. This data base not only permits storage and retrieval of data obtained through the methods of this invention, but also permits acquisition and storage of external data relating to cells. Figure 5 is a PC display which illustrates the operation of the software.

In an alternative embodiment, a high throughput system (HTS) is directly coupled with the HCS either on the same platform or on two separate platforms connected electronically (e.g. via a local area network). This embodiment of the invention, referred to as a dual mode optical system, has the advantage of increasing the throughput of a HCS by coupling it with a HTS and thereby requiring slower high resolution data acquisition and analysis only on the small subset of wells that show a response in the coupled HTS.

High throughput 'whole plate' reader systems are well known in the art and are commonly used as a component of an HTS system used to screen large numbers of compounds (Beggs (1997), *J. of Biomolec. Screening* 2:71-78; Macaffrey et al., (1996) *J. Biomolec. Screening* 1:187-190).

In one embodiment of dual mode cell based screening, a two platform architecture in which high throughput acquisition occurs on one platform and high content acquisition occurs on a second platform is provided (Figure 6). Processing occurs on each platform independently, with results passed over a network interface, or a single controller is used to process the data from both platforms.

As illustrated in Figure 6, an exemplified two platform dual mode optical system consists of two light optical instruments, a high throughput platform 60 and a high content platform 65, which read fluorescent signals emitted from cells cultured in microtiter plates or microwell arrays on a microplate, and communicate with each other via an electronic connection 64. The high throughput platform 60 analyzes all the wells in the whole plate either in parallel or rapid serial fashion. Those skilled in the art of screening will recognize that there are a many such commercially available high throughput reader systems that could be integrated into a dual mode cell based screening system (Topcount (Packard Instruments, Meriden, CT); Spectramax, Lumiskan (Molecular Devices, Sunnyvale, CA); Fluoroscan

(Labsystems, Beverly, MA)). The high content platform 65, as described above, scans from well to well and acquires and analyzes high resolution image data collected from individual cells within a well.

The HTS software, residing on the system's computer 62, controls the high throughput instrument, and results are displayed on the monitor 61. The HCS software, residing on it's computer system 67, controls the high content instrument hardware 65, optional devices (e.g. plate loader, environmental chamber, fluid dispenser), analyzes digital image data from the plate, displays results on the monitor 66 and manages data measured in an integrated database. The two systems can also share a single computer, in which case all data would be collected, processed and displayed on that computer, without the need for a local area network to transfer the data. Microtiter plates are transferred from the high throughput system to the high content system 63 either manually or by a robotic plate transfer device, as is well known in the art (Beggs (1997), *supra*; Mcaffrey (1996), *supra*).

In a preferred embodiment, the dual mode optical system utilizes a single platform system (Figure 7). It consists of two separate optical modules, an HCS module 203 and an HTS module 209 that can be independently or collectively moved so that only one at a time is used to collect data from the microtiter plate 201. The microtiter plate 201 is mounted in a motorized X,Y stage so it can be positioned for imaging in either HTS or HCS mode. After collecting and analyzing the HTS image data as described below, the HTS optical module 209 is moved out of the optical path and the HCS optical module 203 is moved into place.

The optical module for HTS 209 consists of a projection lens 214, excitation wavelength filter 213 and dichroic mirror 210 which are used to illuminate the whole bottom of the plate with a specific wavelength band from a conventional microscope lamp system (not illustrated). The fluorescence emission is collected through the dichroic mirror 210 and emission wavelength filter 211 by a lens 212 which forms an image on the camera 216 with sensor 215.

The optical module for HCS 203 consists of a projection lens 208, excitation wavelength filter 207 and dichroic mirror 204 which are used to illuminate the back aperture of the microscope objective 202, and thereby the field of that objective, from a standard microscope illumination system (not shown). The fluorescence emission is collected by the microscope objective 202, passes through the dichroic mirror 204 and emission wavelength filter 205 and is focused by a tube lens 206 which forms an image on the same camera 216 with sensor 215.

In an alternative embodiment of the present invention, the cell screening system further comprises a fluid delivery device for use with the live cell embodiment of the method of cell screening (see below). Figure 8 exemplifies a fluid delivery device for use with the system of the invention. It consists of a bank of 12 syringe pumps 701 driven by a single motor drive. Each syringe 702 is sized according to the volume to be delivered to each well, typically between 1 and 100 µL. Each syringe is attached via flexible tubing 703 to a similar bank of connectors which accept standard pipette tips 705. The bank of pipette tips are attached to a drive system so they can be lowered and raised relative to the microtiter plate 706 to deliver fluid to each well. The plate is mounted on an X,Y stage, allowing movement relative to the optical system 707 for data collection purposes. This set-up allows one set of pipette tips, or even a single pipette tip, to deliver reagent to all the wells on the plate. The bank of syringe pumps can be used to deliver fluid to 12 wells simultaneously, or to fewer wells by removing some of the tips.

In another aspect, the present invention provides a method for analyzing cells comprising providing an array of locations which contain multiple cells wherein the cells contain one or more fluorescent reporter molecules; scanning multiple cells in each of the locations containing cells to obtain fluorescent signals from the fluorescent reporter molecule in the cells; converting the fluorescent signals into digital data; and utilizing the digital data to determine the distribution, environment or activity of the fluorescent reporter molecule within the cells.

Cell Arrays

Screening large numbers of compounds for activity with respect to a particular biological function requires preparing arrays of cells for parallel handling of cells and reagents. Standard 96 well microtiter plates which are 86 mm by 129 mm, with 6mm diameter wells on a 9mm pitch, are used for compatibility with current automated loading and robotic handling systems. The microplate is typically 20 mm by 30 mm, with cell locations that are 100-200 microns in dimension on a pitch of about 500 microns. Methods for making microplates are described in U.S. Patent Application Serial No. 08/865,341, incorporated by reference herein in its entirety. Microplates may consist of coplanar layers of materials to which cells adhere, patterned with materials to which cells will not adhere, or etched 3-dimensional surfaces of similarly pattered materials. For the purpose of the following discussion, the terms 'well' and 'microwell' refer to a location in an array of any construction

to which cells adhere and within which the cells are imaged. Microplates may also include fluid delivery channels in the spaces between the wells. The smaller format of a microplate increases the overall efficiency of the system by minimizing the quantities of the reagents, storage and handling during preparation and the overall movement required for the scanning operation. In addition, the whole area of the microplate can be imaged more efficiently, allowing a second mode of operation for the microplate reader as described later in this document.

Fluorescence Reporter Molecules

A major component of the new drug discovery paradigm is a continually growing family of fluorescent and luminescent reagents that are used to measure the temporal and spatial distribution, content, and activity of intracellular ions, metabolites, macromolecules, and organelles. Classes of these reagents include labeling reagents that measure the distribution and amount of molecules in living and fixed cells, environmental indicators to report signal transduction events in time and space, and fluorescent protein biosensors to measure target molecular activities within living cells. A multiparameter approach that combines several reagents in a single cell is a powerful new tool for drug discovery.

The method of the present invention is based on the high affinity of fluorescent or luminescent molecules for specific cellular components. The affinity for specific components is governed by physical forces such as ionic interactions, covalent bonding (which includes chimeric fusion with protein-based chromophores, fluorophores, and lumiphores), as well as hydrophobic interactions, electrical potential, and, in some cases, simple entrapment within a cellular component. The luminescent probes can be small molecules, labeled macromolecules, or genetically engineered proteins, including, but not limited to green fluorescent protein chimeras.

Those skilled in this art will recognize a wide variety of fluorescent reporter molecules that can be used in the present invention, including, but not limited to, fluorescently labeled biomolecules such as proteins, phospholipids and DNA hybridizing probes. Similarly, fluorescent reagents specifically synthesized with particular chemical properties of binding or association have been used as fluorescent reporter molecules (Barak et al., (1997), *J. Biol. Chem.* 272:27497-27500; Southwick et al., (1990), *Cytometry* 11:418-430; Tsien (1989) in *Methods in Cell Biology*, Vol. 29 Taylor and Wang (eds.), pp. 127-156). Fluorescently labeled antibodies are particularly useful reporter molecules due to their high degree of

specificity for attaching to a single molecular target in a mixture of molecules as complex as a cell or tissue.

The luminescent probes can be synthesized within the living cell or can be transported into the cell via several non-mechanical modes including diffusion, facilitated or active transport, signal-sequence-mediated transport, and endocytotic or pinocytotic uptake. Mechanical bulk loading methods, which are well known in the art, can also be used to load luminescent probes into living cells (Barber et al. (1996), *Neuroscience Letters* 207:17-20; Bright et al. (1996), *Cytometry* 24:226-233; McNeil (1989) in *Methods in Cell Biology*, Vol. 29, Taylor and Wang (eds.), pp. 153-173). These methods include electroporation and other mechanical methods such as scrape-loading, bead-loading, impact-loading, syringe-loading, hypertonic and hypotonic loading. Additionally, cells can be genetically engineered to express reporter molecules, such as GFP, coupled to a protein of interest as previously described (Chalfie and Prasher U.S. Patent No. 5,491,084; Cubitt et al. (1995), *Trends in Biochemical Science* 20:448-455).

Once in the cell, the luminescent probes accumulate at their target domain as a result of specific and high affinity interactions with the target domain or other modes of molecular targeting such as signal-sequence-mediated transport. Fluorescently labeled reporter molecules are useful for determining the location, amount and chemical environment of the reporter. For example, whether the reporter is in a lipophilic membrane environment or in a more aqueous environment can be determined (Giuliano et al. (1995), *Ann. Rev. of Biophysics and Biomolecular Structure* 24:405-434; Giuliano and Taylor (1995), *Methods in Neuroscience* 27:1-16). The pH environment of the reporter can be determined (Bright et al. (1989), *J. Cell Biology* 104:1019-1033; Giuliano et al. (1987), *Anal. Biochem.* 167:362-371; Thomas et al. (1979), *Biochemistry* 18:2210-2218). It can be determined whether a reporter having a chelating group is bound to an ion, such as Ca++, or not (Bright et al. (1989), In *Methods in Cell Biology*, Vol. 30, Taylor and Wang (eds.), pp. 157-192; Shimoura et al. (1988), *J. of Biochemistry* (Tokyo) 251:405-410; Tsien (1989) In *Methods in Cell Biology*, Vol. 30, Taylor and Wang (eds.), pp. 127-156).

Furthermore, certain cell types within an organism may contain components that can be specifically labeled that may not occur in other cell types. For example, epithelial cells often contain polarized membrane components. That is, these cells asymmetrically distribute macromolecules along their plasma membrane. Connective or supporting tissue cells often contain granules in which are trapped molecules specific to that cell type (e.g., heparin,

histamine, serotonin, etc.). Most muscular tissue cells contain a sarcoplasmic reticulum, a specialized organelle whose function is to regulate the concentration of calcium ions within the cell cytoplasm. Many nervous tissue cells contain secretory granules and vesicles in which are trapped neurohormones or neurotransmitters. Therefore, fluorescent molecules can be designed to label not only specific components within specific cells, but also specific cells within a population of mixed cell types.

Those skilled in the art will recognize a wide variety of ways to measure fluorescence. For example, some fluorescent reporter molecules exhibit a change in excitation or emission spectra, some exhibit resonance energy transfer where one fluorescent reporter loses fluorescence, while a second gains in fluorescence, some exhibit a loss (quenching) or appearance of fluorescence, while some report rotational movements (Giuliano et al. (1995), *Ann. Rev. of Biophysics and Biomol. Structure* 24:405-434; Giuliano et al. (1995), *Methods in Neuroscience* 27:1-16).

Scanning cell arrays

Referring to Figure 9, a preferred embodiment is provided to analyze cells that comprises operator-directed parameters being selected based on the assay being conducted, data acquisition by the cell screening system on the distribution of fluorescent signals within a sample, and interactive data review and analysis. At the start of an automated scan the operator enters information 100 that describes the sample, specifies the filter settings and fluorescent channels to match the biological labels being used and the information sought, and then adjusts the camera settings to match the sample brightness. For flexibility to handle a range of samples, the software allows selection of various parameter settings used to identify nuclei and cytoplasm, and selection of different fluorescent reagents, identification of cells of interest based on morphology or brightness, and cell numbers to be analyzed per well. These parameters are stored in the system's database for easy retrieval for each automated run. The system's interactive cell identification mode simplifies the selection of morphological parameter limits such as the range of size, shape, and intensity of cells to be analyzed. The user specifies which wells of the plate the system will scan and how many fields or how many cells to analyze in each well. Depending on the setup mode selected by the user at step 101, the system either automatically pre-focuses the region of the plate to be scanned using an autofocus procedure to "find focus" of the plate 102 or the user interactively pre-focuses 103 the scanning region by selecting three "tag" points which define the rectangular area to be

scanned. A least-squares fit "focal plane model" is then calculated from these tag points to estimate the focus of each well during an automated scan. The focus of each well is estimated by interpolating from the focal plane model during a scan.

During an automated scan, the software dynamically displays the scan status, including the number of cells analyzed, the current well being analyzed, images of each independent wavelength as they are acquired, and the result of the screen for each well as it is determined. The plate 4 (Figure 1) is scanned in a serpentine style as the software automatically moves the motorized microscope XY stage 3 from well to well and field to field within each well of a 96-well plate. Those skilled in the programming art will recognize how to adapt software for scanning of other microplate formats such as 24, 48, and 384 well plates. The scan pattern of the entire plate as well as the scan pattern of fields within each well are programmed. The system adjusts sample focus with an autofocus procedure 104 (Figure 9) through the Z axis focus drive 5, controls filter selection via a motorized filter wheel 19, and acquires and analyzes images of up to four different colors ("channels" or "wavelengths").

The autofocus procedure is called at a user selected frequency, typically for the first field in each well and then once every 4 to 5 fields within each well. The autofocus procedure calculates the starting Z-axis point by interpolating from the pre-calculated plane focal model. Starting a programmable distance above or below this set point, the procedure moves the mechanical Z-axis through a number of different positions, acquires an image at each position, and finds the maximum of a calculated focus score that estimates the contrast of each image. The Z position of the image with the maximum focus score determines the best focus for a particular field. Those skilled in the art will recognize this as a variant of automatic focusing algorithms as described in Harms et al. in *Cytometry* 5 (1984), 236-243, Groen et al. in *Cytometry* 6 (1985), 81-91, and Firestone et al. in *Cytometry* 12 (1991), 195-206.

For image acquisition, the camera's exposure time is separately adjusted for each dye to ensure a high-quality image from each channel. Software procedures can be called, at the user's option, to correct for registration shifts between wavelengths by accounting for linear (X and Y) shifts between wavelengths before making any further measurements. The electronic shutter 18 is controlled so that sample photo-bleaching is kept to a minimum. Background shading and uneven illumination can be corrected by the software using methods known in the art (Bright et al. (1987), *J. Cell Biol.* 104:1019-1033).

In one channel, images are acquired of a primary marker 105 (Figure 9) (typically cell nuclei counterstained with DAPI or PI fluorescent dyes) which are segmented ("identified") using an adaptive thresholding procedure. The adaptive thresholding procedure 106 is used to dynamically select the threshold of an image for separating cells from the background. The staining of cells with fluorescent dyes can vary to an unknown degree across cells in a microtiter plate sample as well as within images of a field of cells within each well of a microtiter plate. This variation can occur as a result of sample preparation and/or the dynamic nature of cells. A global threshold is calculated for the complete image to separate the cells from background and account for field to field variation. These global adaptive techniques are variants of those described in the art. (Kittler et al. in *Computer Vision, Graphics, and Image Processing* 30 (1985), 125-147, Ridler et al. in *IEEE Trans. Systems, Man, and Cybernetics* (1978), 630-632.)

An alternative adaptive thresholding method utilizes local region thresholding in contrast to global image thresholding. Image analysis of local regions leads to better overall segmentation since staining of cell nuclei (as well as other labeled components) can vary across an image. Using this global/local procedure, a reduced resolution image (reduced in size by a factor of 2 to 4) is first globally segmented (using adaptive thresholding) to find regions of interest in the image. These regions then serve as guides to more fully analyze the same regions at full resolution. A more localized threshold is then calculated (again using adaptive thresholding) for each region of interest.

The output of the segmentation procedure is a binary image wherein the objects are white and the background is black. This binary image, also called a mask in the art, is used to determine if the field contains objects 107. The mask is labeled with a blob labeling algorithm whereby each object (or blob) has a unique number assigned to it. Morphological features, such as area and shape, of the blobs are used to differentiate blobs likely to be cells from those that are considered artifacts. The user pre-sets the morphological selection criteria by either typing in known cell morphological features or by using the interactive training utility. If objects of interest are found in the field, images are acquired for all other active channels 108, otherwise the stage is advanced to the next field 109 in the current well. Each object of interest is located in the image for further analysis 110. The software determines if the object meets the criteria for a valid cell nucleus 111 by measuring its morphological features (size and shape). For each valid cell, the XYZ stage location is recorded, a small image of the cell is stored, and features are measured 112.

The cell scanning method of the present invention can be used to perform many different assays on cellular samples by applying a number of analytical methods simultaneously to measure features at multiple wavelengths. An example of one such assay provides for the following measurements:

1. The total fluorescent intensity within the cell nucleus for colors 1-4
2. The area of the cell nucleus for color 1 (the primary marker)
3. The shape of the cell nucleus for color 1 is described by three shape features:
   a) perimeter squared area
   b) box area ratio
   c) height width ratio
4. The average fluorescent intensity within the cell nucleus for colors 1-4 (i.e. #1 divided by #2)
5. The total fluorescent intensity of a ring outside the nucleus (see Figure 10) that represents fluorescence of the cell's cytoplasm (cytoplasmic mask) for colors 2-4
6. The area of the cytoplasmic mask
7. The average fluorescent intensity of the cytoplasmic mask for colors 2-4 (i.e. #5 divided by #6)
8. The ratio of the average fluorescent intensity of the cytoplasmic mask to average fluorescent intensity within the cell nucleus for colors 2-4 (i.e. #7 divided by #4)
9. The difference of the average fluorescent intensity of the cytoplasmic mask and the average fluorescent intensity within the cell nucleus for colors 2-4 (i.e. #7 minus #4)
10. The number of fluorescent domains (also call spots, dots, or grains) within the cell nucleus for colors 2-4

Features 1 through 4 are general features of the different cell screening assays of the invention. These steps are commonly used in a variety of image analysis applications and are well known in art (Russ (1992) *The Image Processing Handbook*, CRC Press Inc.; Gonzales et al. (1987), *Digital Image Processing*. Addison-Wesley Publishing Co. pp. 391-448). Features 5-9 have been developed specifically to provide measurements of a cell's fluorescent molecules within the local cytoplasmic region of the cell and the translocation (i.e. movement) of fluorescent molecules from the cytoplasm to the nucleus. These features (steps 5-9) are used for analyzing cells in microplates for the inhibition of nuclear translocation. For example, inhibition of nuclear translocation of transcription factors provides a novel approach to screening intact cells (detailed examples of other types of screens will be provided below). A specific algorithm measures the amount of probe in the nuclear region (feature 4) versus the local cytoplasmic region (feature 7) of each cell. Quantification of the difference between these two sub-cellular compartments provides a measure of cytoplasm-nuclear translocation (feature 9).

Feature 10 describes a screen used for counting of DNA or RNA probes within the nuclear region in colors 2-4. For example, probes are commercially available for identifying chromosome-specific DNA sequences (Life Technologies, Gaithersburg, MD; Genosys, Woodlands, TX; Biotechnologies, Inc., Richmond, CA; Bio 101, Inc., Vista, CA) Cells are three-dimensional in nature and when examined at a high magnification under a microscope one probe may be in-focus while another may be completely out-of-focus. The cell screening method of the present invention provides for detecting three-dimensional probes in nuclei by acquiring images from multiple focal planes. The software moves the Z-axis motor drive 5 (Figure 1) in small steps where the step distance is user selected to account for a wide range of different nuclear diameters. At each of the focal steps, an image is acquired. The maximum gray-level intensity from each pixel in each image is found and stored in a resulting maximum projection image. The maximum projection image is then used to count the probes. The above algorithm works well in counting probes that are not stacked directly above or below another one. To account for probes stacked on top of each other in the Z-direction, users can select an option to analyze probes in each of the focal planes acquired. In this mode, the scanning system performs the maximum plane projection algorithm as discussed above, detects probe regions of interest in this image, then further analyzes these regions in all the focal plane images.

After measuring cell features 112 (Figure 9), the system checks if there are any unprocessed objects in the current field 113. If there are any unprocessed objects, it locates the next object 110 and determines whether it meets the criteria for a valid cell nucleus 111, and measures its features. Once all the objects in the current field are processed, the system determines whether analysis of the current plate is complete 114; if not, it determines the need to find more cells in the current well 115. If the need exists, the system advances the XYZ stage to the next field within the current well 109 or advances the stage to the next well 116 of the plate.

After a plate scan is complete, images and data can be reviewed with the system's image review, data review, and summary review facilities. All images, data, and settings from a scan are archived in the system's database for later review or for interfacing with a network information management system. Data can also be exported to other third-party statistical packages to tabulate results and generate other reports. Users can review the images alone of every cell analyzed by the system with an interactive image review procedure 117. The user can review data on a cell-by-cell basis using a combination of interactive graphs, a data

spreadsheet of measured features, and images of all the fluorescence channels of a cell of interest with the interactive cell-by-cell data review procedure 118. Graphical plotting capabilities are provided in which data can be analyzed via interactive graphs such as histograms and scatter plots. Users can review summary data that are accumulated and summarized for all cells within each well of a plate with an interactive well-by-well data review procedure 119. Hard copies of graphs and images can be printed on a wide range of standard printers.

As a final phase of a complete scan, reports can be generated on one or more statistics of the measured features. Users can generate a graphical report of data summarized on a well-by-well basis for the scanned region of the plate using an interactive report generation procedure 120. This report includes a summary of the statistics by well in tabular and graphical format and identification information on the sample. The report window allows the operator to enter comments about the scan for later retrieval. Multiple reports can be generated on many statistics and be printed with the touch of one button. Reports can be previewed for placement and data before being printed.

The above-recited embodiment of the method operates in a single high resolution mode referred to as the high content screening (HCS) mode. The HCS mode provides sufficient spatial resolution within a well (on the order of 1 μm) to define the distribution of material within the well, as well as within individual cells in the well. The high degree of information content accessible in that mode, comes at the expense of speed and complexity of the required signal processing.

In an alternative embodiment, a high throughput system (HTS) is directly coupled with the HCS either on the same platform or on two separate platforms connected electronically (e.g. via a local area network). This embodiment of the invention, referred to as a dual mode optical system, has the advantage of increasing the throughput of an HCS by coupling it with an HTS and thereby requiring slower high resolution data acquisition and analysis only on the small subset of wells that show a response in the coupled HTS.

High throughput 'whole plate' reader systems are well known in the art and are commonly used as a component of an HTS system used to screen large numbers of compounds (Beggs et al. (1997), *supra*; McCaffrey et al. (1996), *supra*). The HTS of the present invention is carried out on the microtiter plate or microwell array by reading many or all wells in the plate simultaneously with sufficient resolution to make determinations on a well-by-well basis. That is, calculations are made by averaging the total signal output of many

or all the cells or the bulk of the material in each well. Wells that exhibit some defined response in the HTS (the 'hits') are flagged by the system. Then on the same microtiter plate or microwell array, each well identified as a hit is measured via HCS as described above. Thus, the dual mode process involves:

1. Rapidly measuring numerous wells of a microtiter plate or microwell array,
2. Interpreting the data to determine the overall activity of fluorescently labeled reporter molecules in the cells on a well-by-well basis to identify "hits" (wells that exhibit a defined response),
3. Imaging numerous cells in each "hit" well, and
4. Interpreting the digital image data to determine the distribution, environment or activity of the fluorescently labeled reporter molecules in the individual cells (i.e. intracellular measurements) and the distribution of the cells to test for specific biological functions

In a preferred embodiment of dual mode processing (Figure 11), at the start of a run 301, the operator enters information 302 that describes the plate and its contents, specifies the filter settings and fluorescent channels to match the biological labels being used, the information sought and the camera settings to match the sample brightness. These parameters are stored in the system's database for easy retrieval for each automated run. The microtiter plate or microwell array is loaded into the cell screening system 303 either manually or automatically by controlling a robotic loading device. An optional environmental chamber 304 is controlled by the system to maintain the temperature, humidity and CO_2 levels in the air surrounding live cells in the microtiter plate or microwell array. An optional fluid delivery device 305 (see Figure 8) is controlled by the system to dispense fluids into the wells during the scan.

High throughput processing 306 is first performed on the microtiter plate or microwell array by acquiring and analyzing the signal from each of the wells in the plate. The processing performed in high throughput mode 307 is illustrated in Figure 12 and described below. Wells that exhibit some selected intensity response in this high throughput mode ("hits") are identified by the system. The system performs a conditional operation 308 that tests for hits. If hits are found, those specific hit wells are further analyzed in high content (micro level) mode 309. The processing performed in high content mode 312 is illustrated in Figure 13. The system then updates 310 the informatics database 311 with results of the measurements on the plate. If there are more plates to be analyzed 313 the system loads the next plate 303; otherwise the analysis of the plates terminates 314.

The following discussion describes the high throughput mode illustrated in Figure 12. The preferred embodiment of the system, the single platform dual mode screening system,

will be described. Those skilled in the art will recognize that operationally the dual platform system simply involves moving the plate between two optical systems rather than moving the optics. Once the system has been set up and the plate loaded, the system begins the HTS acquisition and analysis 401. The HTS optical module is selected by controlling a motorized optical positioning device 402 on the dual mode system. In one fluorescence channel, data from a primary marker on the plate is acquired 403 and wells are isolated from the plate background using a masking procedure 404. Images are also acquired in other fluorescence channels being used 405. The region in each image corresponding to each well 406 is measured 407. A feature calculated from the measurements for a particular well is compared with a predefined threshold or intensity response 408, and based on the result the well is either flagged as a "hit" 409 or not. The locations of the wells flagged as hits are recorded for subsequent high content mode processing. If there are wells remaining to be processed 410 the program loops back 406 until all the wells have been processed 411 and the system exits high throughput mode.

Following HTS analysis, the system starts the high content mode processing 501 defined in Figure 13. The system selects the HCS optical module 502 by controlling the motorized positioning system. For each "hit" well identified in high throughput mode, the XY stage location of the well is retrieved from memory or disk and the stage is then moved to the selected stage location 503. The autofocus procedure 504 is called for the first field in each hit well and then once every 5 to 8 fields within each well. In one channel, images are acquired of the primary marker 505 (typically cell nuclei counterstained with DAPI, Hoechst or PI fluorescent dye). The images are then segmented (separated into regions of nuclei and non-nuclei) using an adaptive thresholding procedure 506. The output of the segmentation procedure is a binary mask wherein the objects are white and the background is black. This binary image, also called a mask in the art, is used to determine if the field contains objects 507. The mask is labeled with a blob labeling algorithm whereby each object (or blob) has a unique number assigned to it. If objects are found in the field, images are acquired for all other active channels 508, otherwise the stage is advanced to the next field 514 in the current well. Each object is located in the image for further analysis 509. Morphological features, such as area and shape of the objects, are used to select objects likely to be cell nuclei 510, and discard (do no further processing on) those that are considered artifacts. For each valid cell nucleus, the XYZ stage location is recorded, a small image of the cell is stored, and assay specific features are measured 511. The system then performs multiple tests on the cells by

applying several analytical methods to measure features at each of several wavelengths. After measuring the cell features, the systems checks if there are any unprocessed objects in the current field 512. If there are any unprocessed objects, it locates the next object 509 and determines whether it meets the criteria for a valid cell nucleus 510, and measures its features. After processing all the objects in the current field, the system determines whether it needs to find more cells or fields in the current well 513. If it needs to find more cells or fields in the current well it advances the XYZ stage to the next field within the current well 515. Otherwise, the system checks whether it has any remaining hit wells to measure 515. If so, it advances to the next hit well 503 and proceeds through another cycle of acquisition and analysis, otherwise the HCS mode is finished 516.

In an alternative embodiment of the present invention, a method of kinetic live cell screening is provided. The previously described embodiments of the invention are used to characterize the spatial distribution of cellular components at a specific point in time, the time of chemical fixation. As such, these embodiments have limited utility for implementing kinetic based screens, due to the sequential nature of the image acquisition, and the amount of time required to read all the wells on a plate. For example, since a plate can require 30 – 60 minutes to read through all the wells, only very slow kinetic processes can be measured by simply preparing a plate of live cells and then reading through all the wells more than once. Faster kinetic processes can be measured by taking multiple readings of each well before proceeding to the next well, but the elapsed time between the first and last well would be too long, and fast kinetic processes would likely be complete before reaching the last well.

The kinetic live cell extension of the invention enables the design and use of screens in which a biological process is characterized by its kinetics instead of, or in addition to, its spatial characteristics. In many cases, a response in live cells can be measured by adding a reagent to a specific well and making multiple measurements on that well with the appropriate timing. This dynamic live cell embodiment of the invention therefore includes apparatus for fluid delivery to individual wells of the system in order to deliver reagents to each well at a specific time in advance of reading the well. This embodiment thereby allows kinetic measurements to be made with temporal resolution of seconds to minutes on each well of the plate. To improve the overall efficiency of the dynamic live cell system, the acquisition control program is modified to allow repetitive data collection from sub-regions of the plate, allowing the system to read other wells between the time points required for an individual well.

Figure 8 describes an example of a fluid delivery device for use with the live cell embodiment of the invention and is described above. This set-up allows one set of pipette tips 705, or even a single pipette tip, to deliver reagent to all the wells on the plate. The bank of syringe pumps 701 can be used to deliver fluid to 12 wells simultaneously, or to fewer wells by removing some of the tips 705. The temporal resolution of the system can therefore be adjusted, without sacrificing data collection efficiency, by changing the number of tips and the scan pattern as follows. Typically, the data collection and analysis from a single well takes about 5 seconds. Moving from well to well and focusing in a well requires about 5 seconds, so the overall cycle time for a well is about 10 seconds. Therefore, if a single pipette tip is used to deliver fluid to a single well, and data is collected repetitively from that well, measurements can be made with about 5 seconds temporal resolution. If 6 pipette tips are used to deliver fluids to 6 wells simultaneously, and the system repetitively scans all 6 wells, each scan will require 60 seconds, thereby establishing the temporal resolution. For slower processes which only require data collection every 8 minutes, fluids can be delivered to one half of the plate, by moving the plate during the fluid delivery phase, and then repetitively scanning that half of the plate. Therefore, by adjusting the size of the sub-region being scanned on the plate, the temporal resolution can be adjusted without having to insert wait times between acquisitions. Because the system is continuously scanning and acquiring data, the overall time to collect a kinetic data set from the plate is then simply the time to perform a single scan of the plate, multiplied by the number of time points required. Typically, 1 time point before addition of compounds and 2 or 3 time points following addition should be sufficient for screening purposes.

Figure 14 shows the acquisition sequence used for kinetic analysis. The start of processing 801 is configuration of the system, much of which is identical to the standard HCS configuration. In addition, the operator must enter information specific to the kinetic analysis being performed 802, such as the sub-region size, the number of time points required, and the required time increment. A sub-region is a group of wells that will be scanned repetitively in order to accumulate kinetic data. The size of the sub-region is adjusted so that the system can scan a whole sub-region once during a single time increment, thus minimizing wait times. The optimum sub-region size is calculated from the setup parameters, and adjusted if necessary by the operator. The system then moves the plate to the first sub-region 803, and to the first well in that sub-region 804 to acquire the prestimulation (time = 0) time points. The acquisition sequence performed in each well is exactly the same as that required for the

specific HCS being run in kinetic mode. Figure 15 details a flow chart for that processing. All of the steps between the start 901 and the return 902 are identical to those described as steps 504 – 514 in Figure 13.

After processing each well in a sub-region, the system checks to see if all the wells in the sub-region have been processed 806 (Figure 14), and cycles through all the wells until the whole region has been processed. The system then moves the plate into position for fluid addition, and controls fluidic system delivery of fluids to the entire sub-region 807. This may require multiple additions for sub-regions which span several rows on the plate, with the system moving the plate on the X,Y stage between additions. Once the fluids have been added, the system moves to the first well in the sub-region 808 to begin acquisition of time points. The data is acquired from each well 809 and as before the system cycles through all the wells in the sub-region 810. After each pass through the sub-region, the system checks whether all the time points have been collected 811 and if not, pauses 813 if necessary 812 to stay synchronized with the requested time increment. Otherwise, the system checks for additional sub-regions on the plate 814 and either moves to the next sub-region 803 or finishes 815. Thus, the kinetic analysis mode comprises operator identification of sub-regions of the microtiter plate or microwells to be screened, based on the kinetic response to be investigated, with data acquisitions within a sub-region prior to data acquisition in subsequent sub-regions.

Specific Screens

In another aspect of the present invention, a machine readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute procedures for defining the distribution and activity of specific cellular constituents and processes is provided. The computer readable medium includes but is not limited to magnetic disks, optical disks, organic memory, and any other volatile (e.g., Random Access Memory ("RAM")) or non-volatile (e.g., Read-Only Memory ("ROM")) mass storage system readable by the CPU. The computer readable medium includes cooperating or interconnected computer readable medium, which exist exclusively on the processing system or be distributed among multiple interconnected processing systems that may be local or remote to the processing system.

In a preferred embodiment, the cell screening system comprises a high magnification fluorescence optical system with a stage adapted for holding cells and a means for moving the

stage, a digital camera, a light source for receiving and processing the digital data from the digital camera, and a computer means for receiving and processing the digital data from the digital camera. This aspect of the invention comprises programs that instruct the cell screening system to define the distribution and activity of specific cellular constituents and processes, using the luminescent probes, the optical imaging system, and the pattern recognition software of the invention. Preferred embodiments of the machine readable storage medium comprise programs consisting of a set of instructions for causing a cell screening system to execute the procedures set forth in Figures 9, 11, 12, 13, 14, 15, or 28. Another preferred embodiment comprises a program consisting of a set of instructions for causing a cell screening system to execute procedures for detecting the distribution and activity of specific cellular constituents and processes. In most preferred embodiments, the cellular processes include, but are not limited to, nuclear translocation of a protein, cellular hypertrophy, apoptosis, transmembrane receptor internalization, and protease-induced translocation of a protein.

The following examples are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined in the claims appended hereto.

The various chemical compounds, reagents, dyes, and antibodies that are referred to in the following Examples are commercially available from such sources as Sigma Chemical (St. Louis, MO), Molecular Probes (Eugene, OR), Aldrich Chemical Company (Milwaukee, WI), Accurate Chemical Company (Westbury, NY), Jackson Immunolabs, and Clontech (Palo Alto, CA).

Example 1 Automated Screen for Compounds that Induce or Inhibit Nuclear Translocation of a DNA Transcription Factor

Regulation of transcription of some genes involves activation of a transcription factor in the cytoplasm, resulting in that factor being transported into the nucleus where it can initiate transcription of a particular gene or genes. This change in transcription factor distribution is the basis of a screen for the cell-based screening system to detect compounds that inhibit or induce transcription of a particular gene or group of genes. A general description of the screen is given followed by a specific example.

The distribution of the transcription factor is determined by labeling the nuclei with a DNA specific fluorophore like Hoechst 33423 and the transcription factor with a specific fluorescent antibody. After autofocusing on the Hoechst labeled nuclei, an image of the nuclei

is acquired in the cell-based screening system at 20x magnification and used to create a mask by one of several optional thresholding methods, as described *supra*. The morphological descriptors of the regions defined by the mask are compared with the user defined parameters and valid nuclear masks are identified and used with the following algorithm to extract transcription factor distributions. Each valid nuclear mask is eroded to define a slightly smaller nuclear region. The original nuclear mask is then dilated in two steps to define a ring shaped region around the nucleus, which represents a cytoplasmic region. The average antibody fluorescence in each of these two regions is determined, and the difference between these averages is defined as the NucCyt Difference. Two examples of determining nuclear translocation are discussed below and illustrated in Figure 10A-J. Figure 10A illustrates an unstimulated cell with its nucleus 200 labeled with a blue fluorophore and a transcription factor in the cytoplasm 201 labeled with a green fluorophore. Figure 10B illustrates the nuclear mask 202 derived by the cell-based screening system. Figure 10C illustrates the cytoplasm 203 of the unstimulated cell imaged at a green wavelength. Figure 10D illustrates the nuclear mask 202 is eroded (reduced) once to define a nuclear sampling region 204 with minimal cytoplasmic distribution. The nucleus boundary 202 is dilated (expanded) several times to form a ring that is 2-3 pixels wide that is used to define the cytoplasmic sampling region 205 for the same cell. Figure 10E further illustrates a side view which shows the nuclear sampling region 204 and the cytoplasmic sampling region 205. Using these two sampling regions, data on nuclear translocation can be automatically analyzed by the cell-based screening system on a cell by cell basis. Figure 10F-J illustrates the strategy for determining nuclear translocation in a stimulated cell. Figure 10F illustrates a stimulated cell with its nucleus 206 labeled with a blue fluorophore and a transcription factor in the cytoplasm 207 labeled with a green fluorophore. The nuclear mask 208 in Figure 10G is derived by the cell based screening system. Figure 10H illustrates the cytoplasm 209 of a stimulated cell imaged at a green wavelength. Figure 10I illustrates the nuclear sampling region 211 and cytoplasmic sampling region 212 of the stimulated cell. Figure 10J further illustrates a side view which shows the nuclear sampling region 211 and the cytoplasmic sampling region 212.

A specific application of this method has been used to validate this method as a screen. A human cell line was plated in 96 well microtiter plates. Some rows of wells were titrated with agonist, a known inducer of a specific nuclear transcription factor. The cells were then fixed and stained by standard methods with a fluorescein labeled antibody to the

transcription factor, and Hoechst 33423. The cell-based screening system was used to acquire and analyze images from this plate and the NucCyt Difference was found to be strongly correlated with the amount of agonist added to the wells as illustrated in Figure 16. In a second experiment, an antagonist to the receptor for the agonist was titrated in the presence of agonist, progressively inhibiting agonist-induced translocation of the transcription factor. The NucCyt Difference was found to strongly correlate with this inhibition of translocation, as illustrated in Figure 17.

Additional experiments have shown that the NucCyt Difference gives consistent results over a wide range of cell densities and reagent concentrations, and can therefore be routinely used to screen compound libraries for specific nuclear translocation activity. Furthermore, the same method can be used with antibodies to other transcription factors, or GFP-transcription factor chimeras, in living and fixed cells, to screen for effects on the regulation of transcription of this and other genes.

Figure 18 is a representative display on a PC screen of data which was obtained in accordance with Example 1. Graph 1 180 plots the difference between the average antibody fluorescence in the nuclear sampling region and cytoplasmic sampling region, NucCyt Difference verses Well #. Graph 2 181 plots the average fluorescence of the antibody in the nuclear sampling region, NP1 average, versus the Well #. Graph 3 182 plots the average antibody fluorescence in the cytoplasmic sampling region, LIP1 average, versus Well #. The software permits displaying data from each cell. For example, Figure 18 shows a screen display 183, the nuclear image 184, and the fluorescent antibody image 185 for cell #26.

NucCyt Difference referred to in graph 1 180 of Figure 18 is the difference between the average cytoplasmic probe (fluorescent reporter molecule) intensity and the average nuclear probe (fluorescent reporter molecule) intensity. NP1 average referred to in graph 2 181 of Figure 18 is the average of cytoplasmic probe (fluorescent reporter molecule) intensity within the nuclear sampling region. L1P1 average referred to in graph 3 182 of Figure 18 is the average probe (fluorescent reporter molecule) intensity within the cytoplasmic sampling region.

Example 2 Automated Screen for Compounds that Induce or Inhibit Hypertrophy in Cardiac Myocytes

Hypertrophy in cardiac myocytes has been associated with a cascade of alterations in gene expression and can be characterized in cell culture by an alteration in cell size, that is

clearly visible in adherent cells growing on a coverslip. A screen is implemented using the following strategy. Myocyte cell line QM7 (Quail muscle clone 7; ATCC CRL-1962) cultured in 96 well plates, can be treated with various compounds and then fixed and labeled with a fluorescent antibody to a cell surface marker and a DNA label like Hoechst. After focusing on the Hoechst labeled nuclei, two images are acquired, one of the Hoechst labeled nuclei and one of the fluorescent antibody. The nuclei are identified by thresholding to create a mask and then comparing the morphological descriptors of the mask with a set of user defined descriptor values. Local regions containing cells are defined around the nuclei. The limits of the cells in those regions are then defined by a local dynamic threshold operation on the same region in the fluorescent antibody image. A sequence of erosions and dilations is used to separate slightly touching cells and a second set of morphological descriptors is used to identify single cells. The area of the individual cells is tabulated in order to define the distribution of cell sizes for comparison with size data from normal and hypertrophic cells. In addition, a second fluorescent antibody to a particular cellular protein, such as one of the major muscle proteins actin or myosin can be included. Images of this second antibody can be acquired and stored with the above images, for later review, to identify anomalies in the distribution of these proteins in hypertrophic cells, or algorithms can be developed to automatically analyze the distributions of the labeled proteins in these images.

Example 3. Automated Screens for Compounds that Induce or Inhibit Receptor Trafficking Through the Endosomal Systemt

Integral membrane proteins and macromolecules that are endocytosed at the cell surface can have several intracellular fates (Gruenberg and Maxfield, Curr Opin Cell Biol, 1995 7(4): p. 552-63; Mukherjee *et al.*, Physiol Rev, 1997. 77(3): p. 759-803). The major endocytic trafficking pathways deliver recycling receptors back to the plasma membrane via the early endosomal system, and transport soluble volume markers to late endosomes and lysosomes. Internalized receptors that recycle back to the plasma membrane traffic through a compartment known as the recycling endosome or the endocytic recycling compartment. In many cell types (Gruenberg and Maxfield, 1995; Mukherjee et al., Physiol Rev, 1997), recycling compartments that contain fluorescently labeled macromolecules appear as large fluorescent spots in a peri-nuclear location. (Dunn et al., J Cell Biol, 1989. 109(6 Pt 2): p. 3303-14; Mayor et al., J Cell Biol, 1993. 121(6): p. 1257-69; Ghosh *et al.*, J Cell Biol, 1998. 142(4): p. 923-36)

A wide variety of physiologically important macromolecules traffic through or associate with the recycling compartment, such as (1) receptors that constitutively internalize and then recycle, such as the transferrin receptor, and the low-density lipoprotein receptor; (2) many G-protein coupled receptors (GPCRs), that upon agonist stimulation, become desensitized, are internalized into the cell, and then recycle back to the plasma membrane via the recycling compartment as part of their resensitization process (Tarasova *et al.*, J Biol Chem, 1997. 272(23): p. 14817-24; Drmota al., J Biol Chem, 1998. 273(37): p. 24000-8; Kallal *et al.*, J Biol Chem, 1998. 273(1): p. 322-8; Conway *et al.*, Journal of Biomolecular Screening, 1999. 4(2): p. 75-86); (3) various lipids and lipid analogs (Mayor et al.,. J Cell Biol, 1993. 121(6): p. 1257-69; Mukherjee et al., J Cell Biol, 1999. 144(6): p. 1271-84); (4) Glycosylphosphatidylinositol (GPI)-anchored proteins (Mayor et al., EMBO J, 1998. 17(16): p. 4626-38); (5) cholesterol and its analogs (Mukherjee *et al.*, Biophys J, 1998. 75(4): p. 1915-25); and (6) various other cytoplasmic signaling and trafficking-related proteins (Ghosh *et al.*, J Cell Biol, 1998. 142(4): p. 923-36; Johnson *et al.*, J Cell Biol, 1996. 135(6 Pt 2): p. 1749-62; Galli, *et al.*, J Cell Biol, 1994. 125(5): p. 1015-24; Daro *et al.*, Proc Natl Acad Sci U S A, 1996. 93(18): p. 9559-64; Ullrich *et al.*, J Cell Biol, 1996. 135(4): p. 913-24). Receptors that internalize and then recycle back to the plasma membrane can be classified into two groups: (1) receptors that constitutively internalize and then recycle regardless of ligand binding, and (2) receptors that internalize and recycle only upon ligand binding. Among the receptors that constitutively internalize and recycle are those involved in nutrient intake into cells such as the transferrin receptor and the low-density lipoprotein receptor. (Gruenberg and Maxfield, 1995; Mukherjee et al., Physiol Rev, 1997) The GPCRs internalize only after ligand binding and stimulation.

Thus, in another aspect, the present invention provides fully automated methods for measuring and analyzing macromolecule trafficking through or association with the cell endosomal system. As used herein, the term "endosomal system" includes all components of endocytic trafficking pathways, including early endosomes, late endosomes, recycling endosomes (also referred to as the endocytic recycling compartment), and lysosomes. As used herein, the term "trafficking through the endosomal system" includes both macromolecule movement into the endosomal system from elsewhere on or in the cell, macromolecule recycling of macromolecules from the endosomal system to elsewhere on or in the cell, and macromolecule association with components of the endocytic trafficking pathways).

In one aspect of the present invention, methods, computer readable storage medium, and kits are provided for identifying compounds that induce or inhibit macromolecule trafficking through the endosomal system, comprising treating cells that possess a luminescently-tagged macromolecule with a test compound, obtaining luminescent signals from the cells, converting the luminescent signals into digital data, and utilizing the digital data to determine whether the test compound has induced or inhibited macromolecule trafficking through the endosomal system.

Various preferred embodiments are provided, that allow for improved spatial resolution and quantitation of the stimulatory or inhibitory effect of the test compound on macromolecule trafficking. In another embodiment, combined high throughput and high content methods and associated computer readable storage medium are provided for identifying compounds that induce or inhibit macromolecule trafficking through the endosomal system.

As used herein, the cells "possess a luminescently labeled macromolecule", which includes expression by the cells or addition to the cells either before, together with, or after contacting the cells with a test compound. For example, the macromolecule may be expressed as a luminescently labeled macromolecule by transfected indicator cells. Alternatively, the luminescently labeled macromolecule may be expressed, isolated, and/or bulk-loaded into the cells, or the macromolecule may be luminescently labeled after isolation. As a further alternative, the macromolecule may be expressed by the cells, which are subsequently contacted with a luminescent label, such as an antibody, that detects the macromolecule.

In a preferred embodiment of the present invention, the macromolecule is a protein, and most preferably is a cell surface receptor protein, such as a GPCR. The presence of a GPCR in the endosomal system indicates that it has been stimulated by an agonist, and is trafficking through the recycling compartment as part of its re-sensitization process. Thus, quantifying the presence of fluorescently labeled GPCRs within the recycling compartment is a method of assaying for GPCR activation by agonists, and can also be used to screen for agonists of orphan GPCRs.

In a further preferred embodiment, the cells possess at least a luminescently labeled G protein coupled receptor (GPCR) and a luminescently labeled constitutively internalized receptor, including but not limited to the transferrin receptor. In this embodiment, the method distinguishes between test compounds that are specific agonists or antagonists of the

GPCR, and those that are activators or inhibitors of generalized endocytosis. If the test compound promotes internalization of both receptors, it is an activator of generalized endocytosis. If the test compound promotes internalization of only the GPCR, it is a specific agonist of the GPCR. If the test compound inhibits internalization of both receptors under conditions that promote internalization, it is a general inhibitor of endocytosis. If the test compound inhibits internalization of only the GPCR under conditions that promote specific GPCR internalization, it is a specific antagonist of the GPCR.

In these various embodiments, living cells are obtained from continuous lines of normal or transformed cells, or primary normal or transformed cells obtained directly from animals. Where the macromolecule is a protein, the appropriate cells may be transiently or stably transfected with a DNA construct (either plasmid or viral based) that expresses the protein of interest fused to a molecular based chromophore at either its amino or carboxy terminus or internally such that the protein retains function. Examples of useful molecular-based chromophores include, but are not limited to, GFP and any of its various mutants (Heim and Tsien (1996) Current Biology 6: 178-182; Zhang et al. (1996) Biochem. Biophys. Res. Comm. 227: 707-711). In addition, any of the luciferases and their mutants could also be used. The expression of the chimeric protein may be constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone, steroid-responsive).

Alternatively, the cells are transiently or stably transfected with a DNA construct (either plasmid or viral based) that expresses the protein of interest fused to a small peptide or epitope tag. The epitope tag may be fused to the amino or carboxy terminus, or internally such that the protein remains functional, or, alternatively, the protein may be labeled with two distinct epitope tags, with one being fused to each end of the protein. Some examples of epitope tags include, but are not limited to, FLAG (Sigma Chemical, St. Louis, MO), myc (9E10) (Invitrogen, Carlsbad, CA), 6-His (Invitrogen; Novagen, Madison, WI), and HA (Boehringer Manheim Biochemicals). The expression the GPCR fusion may be constitutive or inducible.

In another embodiment, the cells are transiently or stably transfected with a DNA construct (either plasmid or viral based) that expresses the protein of interest fused to an epitope tag at its amino terminus and a molecular based chromophore at its carboxy terminus. Alternatively, the protein may be fused to an epitope tag at its carboxyl terminus and a

molecular based chromophore at its amino terminus. The expression of the fusion protein may be constitutive or inducible. In another embodiment, fluorescently labeled ligand is used to induce receptor sequestration and the fate of the ligand is followed as a parameter of the high-content screen.

An alternative approach disclosed here involves using dual labeled receptors, comprising a label specific to the amino terminus of the receptor to distinctly label its extracellular domain in addition to a molecular-based chromophore such as GFP or luciferase on the receptor's carboxy terminus to specifically label the intracellular domain. Methods for the construction of such chimeric protein-expressing DNA constructs are well known in the art. (*Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA); *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.).

A ratio of fluorescence intensity of the two labels is made in unstimulated and stimulated cells. Since the amino terminus of the receptor is only available for labeling while the receptor is inserted in the plasma membrane, the ratio of the two labels in unpermeabilized cells can be used to measure the extent of internalization of the receptor.

In another embodiment, cells may contain more than one distinctly labeled macromolecule such that different macromolecules can be analyzed in the same cells by using different fluorescence channels to collect those data. Similarly, the wells or microarrays may contain mixed populations of cells, each population containing a different macromolecule labeled with a spectrally distinct fluorophore, or where each sub-population possesses macromolecules with the same fluorophore, but where the sub-populations of cells are labeled with different second fluorophores to distinguish between the sub-populations. In addition, the luminescently labeled constitutively-internalized receptor can be used as a mask, and it can be determined whether luminescent GPCR co-localizes with it. It is possible to measure the effects of drugs on different macromolecules in a single run by utilizing a cell screening system, such as the cell screening system of the present invention, that is capable of distinguishing the channels of fluorescence of the different macromolecules in these examples. In this way one can screen for compounds that affect multiple macromolecule types or, conversely, for compounds that affect one macromolecule type and not others.

The appropriate cells are then patterned into arrays for treatment and analysis. These arrays can be multiple well plates containing 96, 384, 1536, or more individual wells. The cells can also be arranged into microarrays of "virtual wells" such as those described in U.S. Patent App., Serial No. 08,865,341. These microarrays can be of the same cell type and are treated with a variety of distinct compounds, or alternatively, the microarrays can be a variety of cell types treated with one or more compounds.

Once the chosen cells are patterned into wells or microarrays, they are treated with solutions of drugs or ligands to either inhibit or stimulate macromolecule trafficking through the endosomal system. The fluidic delivery system can be manual, robotic, or via microfluidics such as those described in U.S. Patent App., Serial No. 08,865,341. After an appropriate incubation period, the cells are fixed with a chemical crosslinking agent and stained with luminescence-based reagents. These reagents include, but are not limited to, luminescently labeled primary or secondary antibodies that react with the macromolecule, the epitope tag, or other cellular antigens determined to correlate with macromolecule trafficking through the endosomal system. Biosensors, luminescent stains, dyes, and other small molecules can also be used to measure the physiological response of the cells to drugs. These reagents are used to measure the temporal and spatial changes in ions, metabolites, macromolecules, and organelles induced by drugs.

In another embodiment, cells in wells or microarrays are treated with drugs, and the physiological response is measured temporally and spatially within a population of single living cells after an appropriate incubation period. Luminescent stains, dyes, and other small molecules can be used to measure the physiological response of living cells to drugs. Molecular-based chromophores expressed by the cells themselves (such as GFP and its mutants) are particularly suited to live cell measurements. These reagents can be used to measure the temporal and spatial intracellular changes of ions, metabolites, macromolecules, and organelles induced by drugs. Macromolecular-based indicators of cellular physiology can also be used in the assay. These luminescent analogs and biosensors can be used to measure the temporal and spatial changes in the distribution and activity of the macromolecules in response to drug treatments.

These methods can be applied to any macromolecule that trafficks through the endosomal system and is located in a distinct, microscopically resolvable component of the endosomal system that appears as a distinct spot or object. One major class of macromolecules that the invention can be applied to is cell surface receptor proteins, such as

GPCR. Some known examples of GPCRs are the adrenergic receptors; muscarinic acetylcholine receptors; opioid receptors; chemokine receptors; neuropeptide receptors; prostaglandin receptors; parathyroid hormone receptor; cholecystokinin receptor; secretin receptor; rhodopsin; dopamine receptors; serotonin receptors; odorant receptors; histamine receptors; angiotensin receptors; gastrin receptors; follicle stimulating hormone receptor; luteinizing hormone receptor; metabotropic glutamate receptors; glucagon receptor (a more complete list of known GPCRs and their ligands can be found in Beck-Sickinger, A.G (1996) *Drug Discovery Today* 1(12): 502-513). This aspect of the invention is not limited to GPCRs; examples of other receptors to which this invention could be applied include, but are not limited to, growth factor receptors such as PDGF (Heldin et al. (1982) J. Biol. Chem. 257(8): 4216-4221; Kapeller et al. (1993) Mol. Cell. Biol. 13(10): 6052-6063) and EGF (Zidovetzki et al. (1981) Proc. Natl. Acad. Sci. 78(11): 6981-6985; Beguinot et al. (1984) Proc. Natl. Acad. Sci. 81(8): 2384-2388; Emlet et al. (1997) J. Biol. Chem. 272(7): 4079-4086), the transferrin receptor (Klausner et al. (1983) J. Biol. Chem. 258(8): 4715-4724; Ciechanover et al. (1983) J. Cell. Biochem. 23(1-4): 107-130), and the insulin receptor (Baldwin et al. (1980) Proc. Natl. Acad. Sci. 77(10): 5975-5978; Di Guglielmo et al. (1998) Mol. Cell. Biochem. 182(1-2): 59-63). This invention can also be applied to orphan receptors for which a specific ligand and/or effector is unknown, receptor ligands, lipids and lipid analogs, glycosylphosphatidyl inositol-anchored proteins, cholesterol and its analogs, and cytoplasmic signaling and trafficking proteins.

G-protein coupled receptors

G-protein coupled receptors (GPCRs) are a large class of 7 transmembrane-domain cell surface receptors that transmit signals from the extracellular environment to the cell cytoplasm via their interaction with heterotrimeric G-proteins. Activation of these receptors by ligand binding promotes the exchange of GDP for GTP on the associated G-protein, resulting in the dissociation of the G-protein into active Gα-GTP and Gβγ subunits. The interaction of these subunits with their effectors stimulates a cascade of secondary signals in the cell, such as the production of cyclic AMP (cAMP) and inositol triphosphate (IP_3), Ca^{++} mobilization, and activation of a variety of kinases. A wide range of biological functions are associated with GPCRs, including, but not limited to, smell, taste, perception of light, control of blood pressure, neurotransmission, endocrine and exocrine function, chemotaxis, exocytosis, embryogenesis and development, cell growth and differentiation, and

oncogenesis. GPCRs have therefore become a major potential target for a variety of therapeutic units.

GPCRs span the plasma membrane and undergo a relatively slow rate of endocytosis from the cell surface to endosomes in unstimulated cells. Although poorly understood mechanistically, it is known that the presence of agonist increases the rate of receptor internalization dramatically. Once internalized in endosomes, GPCRs may either be recycled back to the plasma membrane or targeted to lysosomes for degradation. The significance of this sequestration of GPCRs is not yet fully understood. Receptor internalization may play a role in desensitization (loss of functional response) exhibited as a reduction in the ability of the receptor to generate second messenger in the presence of continued stimulation. However, the rate of receptor loss from the surface is usually too slow to account for the rapid rate of desensitization (Tobin, A.B. et al. (1992) *Mol. Pharmacol.* 42: 1042-1048), and there are examples where the two processes have been shown to be uncoupled (Baumgold, J. et al. (1989) *Neuropharmacology* 28: 1253-1261; Kanbe, S. et al. (1990) *Biochem. Pharmacol.* 40: 1005-1014).

It is likely that endocytosis of receptors may be involved in re-sensitization (the re-establishment of the ability of the cell to produce second messenger in response to stimulation). It has been demonstrated for the β_2-adrenergic receptor (β_2-AR) that sequestration deficient mutants as well as receptors treated with agents that block sequestration do not resensitize (Yu, S.S. et al. (1993) *J. Biol. Chem.* 268(1): 337-341; Barak, L.S. et al. (1994) *J. Biol. Chem.* 269(4): 2790-2795). For the β_2-AR, agonist stimulation results in receptor phosphorylation by protein kinase A and β_2-adrenergic receptor kinase (β-ARK). Subsequently there is uncoupling of the receptor from its G protein as a result of the recruitment and binding of β-arrestin to the receptor, and internalization of the receptor via clathrin-coated pits is initiated. The acidic endosomal pH favors phosphatase activity, thus enhancing receptor de-phosphorylation (Krueger, K.M. et al. (1997) *J. Biol. Chem.* 272(1): 5-8) and making the receptor available for recycling to the plasma membrane to re-associate with a G-protein. However, re-sensitization of other receptors, such as the M_4 muscarinic receptors, has been shown to be delayed by endocytosis (Bogatekewitsch, G.S. et al. (1996) *Mol. Pharmacol.* 50: 424-429). Despite the fact that the functional importance of receptor internalization may vary between receptor classes, it remains clear that internalization is a significant step in the pathway of receptor activation and function.

The fundamental importance of cellular processes involving GPCRs makes them a significant target for drug screening. The state of the art for monitoring GPCR-ligand interactions and receptor internalization is limited to measurements of a single event (*e.g.*, receptor-ligand interaction or receptor loss from the plasma membrane). Current procedures include measurements of binding of labeled ligand (usually radioactively labeled) to whole cells or isolated membrane fractions (WO/97/04214; von Zastrow and Kobilka, J. Biol. Chem. 269:18448-18452 (1994); Koch et al., J. Biol. Chem. 273:13652-13657 (1998); Tiberi et al., J. Biol. Chem. 271:3771-3778 (1996)), the coincident migration of receptors with various markers into subcellular fractions resolved through centrifugation (Seibold et al., J. Biol. Chem. 273:7637-7642 (1998); Stefan et al., Mol. Biol. Cell. 9:885-899 (1998)), visualization of fluorescently labeled ligand binding to receptors in fixed cells (Tarasova et al., J. Biol. Chem. 272:14817-14824 (1997)), or antibody labeling (either directly or to epitope tags) to identify receptors (von Zastrow and Kobilka, J. Biol. Chem. 269:18448-18452 (1994); Segredo et al. (1997) J. Neurochem. 68: 2395-2404; Krueger et al. (1997) J. Biol. Chem. 272(1): 5-8; Tiberi et al. (1996) J. Biol. Chem. 271(7): 3771-3778)). More recently, green fluorescent protein (GFP)-receptor fusions have been used, which allows visualization of GPCR receptor trafficking in live cells (Kallal, L. et al. (1998) *J. Biol. Chem.* 273(1): 322-328; Tarasova, N.I. et al. (1997) *J. Biol. Chem.* 272(23): 14817-14824). However, this requires confocal imaging to obtain three-dimensional information in order to distinguish whether a receptor has been internalized or has simply moved in the plane of the plasma membrane. Methods have also been disclosed for the identification of GPCRs, their ligands, and compounds that modulate their activity (WO 98/13353 and WO 97/48820). These methods, however, detect G-protein activation indirectly by ligand binding to the receptor and reporter gene activation. Neither method directly labels the receptor or directly measures the internalization of the receptor as an indication of receptor activation.

While existing approaches have provided information and a means of measuring receptor function, there remains a need in the art for a method to directly measure ligand-induced receptor internalization with high spatial and temporal resolution as a measure of receptor activation. An assay that quantifies internalization, recycling or association with the endosomal system can be used to screen for and characterize compounds that affect a variety of physiological processes.

The following example is a screen for activation of a G-protein coupled receptor (GPCR) as detected by the translocation of the GPCR from the plasma membrane to a

proximal nuclear location. This example illustrates how a high throughput screen can be coupled with a high-content screen in the dual mode system for cell based screening.

Figure 19 illustrates a dual mode screen for activation of a GPCR. Cells carrying a stable chimera of the GPCR with a blue fluorescent protein (BFP) are loaded with the acetoxymethylester form of Fluo-3, a cell permeable calcium indicator (green fluorescence) that is trapped in living cells by the hydrolysis of the esters. They are then deposited into the wells of a microtiter plate 601. The wells are then treated with an array of test compounds using a fluid delivery system, and a short sequence of Fluo-3 images of the whole microtiter plate are acquired and analyzed for wells exhibiting a calcium response (i.e., high throughput mode). The images appear like the illustration of the microtiter plate 601 in Figure 19. A small number of wells, such as wells C4 and E9 in the illustration, would fluoresce more brightly due to the Ca^{++} released upon stimulation of the receptors. The locations of wells containing compounds that induced a response 602, would then be transferred to the HCS program and the optics switched for detailed cell by cell analysis of the blue fluorescence for evidence of GPCR translocation to the perinuclear region. The bottom of Figure 19 illustrates the two possible outcomes of the analysis of the high resolution cell data. The camera images a sub-region 604 of the well area 603, producing images of the fluorescent cells 605. In well C4, the uniform distribution of the fluorescence in the cells indicates that the receptor has not internalized, implying that the Ca^{++} response seen was the result of the stimulation of some other signaling system in the cell. The cells in well E9 606 on the other hand, clearly indicate a concentration of the receptor in the perinuclear region clearly indicating the full activation of the receptor. Because only a few hit wells have to be analyzed with high resolution, the overall throughput of the dual mode system can be quite high, comparable to the high throughput system alone.

Transferrin receptor

The transferrin receptor (TfR) transports iron into cells via its ligand transferrin (Tf). Unlike most receptor ligands, Tf remains bound to its receptor during its intracellular trafficking and recycling itinerary. ((Gruenberg and Maxfield, 1995; Mukherjee et al., Physiol Rev, 1997) This allows fluorescently labeled Tf to be used to monitor the internalization and recycling of the TfR.

Sample Preparation: Transferrin Internalization and Trafficking Assays

COS-1 cells were plated in 96-well black clear-bottomed microplates (Packard, Meridian, CT) at a density range of around 10^4 cells/well approximately 18 hours before doing the experiment. The medium used was McCoy 5A with 5% fetal bovine serum and 4 μM deferoxamine mesylate (Sigma). For the experiments, the medium was removed and the cells were rinsed twice with Eagle's Minimum Essential Medium (EMEM). The cells were then incubated at 37°C for 45 minutes with 50 μL of EMEM per well containing 20 μg/mL Alexa™488-transferrin (AxTf; Molecular Probes). The plates were then rinsed twice with PBS (with Ca and Mg). The wells to be analyzed for TfR internalization were fixed at room temperature for 30 minutes with 100 μL/well of 3.7% formaldehyde solution. The fixative solution also contained 10 μg/mL of the nucleic acid stain Hoechst 33342 (Molecular Probes). To prevent internalization, the AxTf incubation was performed in some wells at 4°C instead of 37°C. For the recycling experiments, after the AxTf was removed, the wells were incubated at 37°C for an additional 60 minutes with EMEM containing 10% fetal bovine serum. The cells were then rinsed with PBS and fixed as described above. For the experiments using monodansyl cadaverine (Sigma), the drug was freshly prepared as a 50 mM stock solution with a final working solution of 250-400 μM in EMEM, and cells were incubated with the drug concurrently with the AxTf.

Prepared plates were screened and the plate data was reviewed and exported to a spreadsheet. The image data were also saved and used to corroborate the numerical data.

Evaluation of Assay Performance

To determine how well an assay performed, various statistical parameters were evaluated. This included calculating the signal-to-noise ratio, the signal-to-background ratio, and the Z-factor. If the positive stimulated wells give a mean result of μ_{C+} with a standard-deviation σ_{C+}, and the negative stimulated wells give a mean result of μ_{C-} with a standard-deviation σ_{C-}, then the signal-to-noise ratio is defined as:

$$S/N = \frac{|\mu_{C+} - \mu_{C-}|}{\sqrt{\sigma_{C+}^2 + \sigma_{C-}^2}}$$

and the signal-to-background ratio is defined as:

$$S/B = \frac{\mu_{C+}}{\mu_{C-}}$$

The Z-factor is a dimensionless statistical parameter used to both evaluate an assay for its suitability for screening, and to compare it to other assays. (Zhang et al., Journal of Biomolecular Screening, 1999. 4(2): p. 67-73) It is defined as:

$$Z = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

Dose Response Curve Fitting and IC_{50}/EC_{50} Determination

To determine the IC_{50} (50% inhibitory concentration) or EC_{50} (50% excitatory concentration) of a compound, each column of wells of a 96-well plate was treated with a different compound concentration. The results from the wells with the same compound concentration were averaged and plotted as a function of compound concentration. The data were fit with the function:

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b}$$

where the IC_{50} concentration is the parameter *c*, and the minimum and maximum values are the parameters *a* and *d*.

Results

To assay for TfR internalization, COS-1 cells were grown on microplates having black sides to minimize cross-talk between wells, but optically clear bottoms for inverted imaging. The cells were incubated at 37°C with the AxTf for 45 minutes. This is sufficient time for the AxTf, bound to its receptor, to get internalized and traffic to the recycling compartment. (Mukherjee et al., *Endocytosis.* Physiol Rev, 1997. 77(3): p. 759-803; Ghosh et al.,. J Cell Sci, 1994. 107(Pt 8): p. 2177-89; Ghosh and Maxfield, J Cell Biol, 1995. 128(4): p. 549-61) The cells were then rinsed, fixed and their nuclei labeled with Hoechst 33342. The Hoechst 33342 stains all the nuclei with a bright blue fluorescence, which can be used in a variety of measurements, including a total cell count. The AxTf in the recycling compartment appeared as an intensely staining fluorescent green spot adjacent to each nucleus.

For image analysis, the Hoechst labeled nuclei were recorded in the blue channel, and the transferrin receptor was recorded in the green channel. One cell field per well was imaged using a 10X objective lens. Depending on the cell plating density, these conditions detected between 350 to 800 cells per field.

At least one of the following features are measured for the images in each well: (1) the total number of nuclei (i.e. cells) and their aggregate area; (2) the percentage of cells with fluorescently labeled receptor in the recycling compartment (i.e.: the total number of spots normalized by the number of nuclei); and/or (3) the average and the aggregate areas of the spots; and (4) the average and aggregate integrated intensity of the spots. These parameters are broadly applicable for use with imaging macromolecule trafficking through or association with the endosomal system.

In one experiment, half the wells contained cells with internalized AxTf, and the remaining wells contained non-internalized AxTf. The surface-bound, non-internalized condition was achieved by incubating the cells with AxTf at 4°C instead of 37°C. For the positive wells (internalization), the mean percentage of cells detected with fluorescent spots was 69.6 ± 3.2 (SD). For the negative wells (no internalization), the mean percentage of cells detected with spots was 0.2 ± 0.2 (SD). These results give a high signal-to-noise ratio (21.6) between the internalized and not internalized conditions; thus, this internalization assay provides a significant window between the positive and negative conditions to allow for screening.

To evaluate this assay for its suitability for screening, and to compare it to other assays, its associated Z-factor was calculated (see above). The results gave a Z-factor of 0.852, confirming that this is an "excellent assay" for screening with a large separation band between the negative and positive responses. An assay validation was performed consisting of two maximum signal plates, two minimum signal plates, and two min/max plates, and run on three separate days. The Z-factor values for the min/max plates from this validation run ranged from 0.74 to 0.85, giving a mean Z-factor of 0.79 ± 0.05; all in the "excellent assay" category. From this validation run, the mean signal-to-noise ratio was 15.7 ± 1.6 (SEM), and the mean signal-to-background ratio was 381 ± 65 (SEM). In addition, the well-to-well variation of the maximum signal plates was 5.4%, the plate-to-plate variation was 5.6%, and the day-to-day variation was 5.9%. The high signal-to-noise and signal-to-background ratios and Z-factor, and the low variabilities between wells, plates and days, indicate the robustness of this assay for screening.

The transglutaminase inhibitor, monodansyl cadaverine (MDC), has been long known to impede internalization and trafficking to the recycling compartment (Davies *et al.*, Nature, 1980. 283: p. 162-167). To measure MDC's IC_{50} concentration for TfR internalization, cells on a 96-well plate were incubated with AxTf as before except MDC was included in the AxTf

incubation. Each column of 8 wells on the 96-well plate received a different concentration of MDC. After the AxTf incubation, the cells were treated as before and then the plate was scanned. The percentage of cells containing fluorescent spots was averaged for all 8 wells in each column, and the resulting MDC dose-response curve was plotted and fit (see Materials and Methods). From this curve, the MDC's IC_{50} value for TfR internalization was found to be 276 ± 27 μM. This demonstrates that the present method can be used to determine compound dose-response curves and IC_{50} or EC_{50} (50% excitatory concentration) values.

The present method can also be used to assay trafficking from the recycling compartment back to the plasma membrane. To assay TfR recycling, the cells are first labeled with AxTf for 45 minutes as described above (without the compound or fixative addition). The recycling compartment contains AxTf, and be fluorescently visible. The cells are then incubated in non-fluorescent chase media for approximately one hour; this chases out over 95% of the cellular AxTf. The cells are then fixed and labeled with Hoechst 33342, and are ready to be imaged and analyzed.

In one experiment, half the wells were incubated with AxTf, and the remaining wells were treated under recycling conditions (AxTf incubation followed by an hour incubation in chase media). Under recycling conditions, little staining was observed, consistent with successful TfR recycling from the recycling compartments. The mean percentage of cells containing internalized AxTf was 71.1 ± 7.4 (SD) compared to 1.6 ± 1.7 (SD) for the AxTf chased wells. The signal-to-noise ratio from these results is 9.2, the signal-to-background ratio is 44.4, and the Z-factor is 0.61; these results are indicative of an excellent assay for screening.

Example 4. High-content screen of ligand-induced parathyroid hormone receptor internalization

Plasmid construct. A eukaryotic expression plasmid containing the coding sequence for a humanized GFP mutant (pEGFP-N_2, CLONTECH, Palo Alto, CA) was used to create a GFP-human parathyroid hormone receptor (PTHR, GenBank #L04308) chimera.

Cell preparation. The plasmid construct was used to transfect a human embryonic kidney cell line (HEK 293) (ATCC NO.CRL-1573). Clonal lines stably expressing the GFP-PTHR chimera were established by antibiotic selection with the neomycin analog Geneticin (0.5 mg/ml; Life Technologies, Gaithersburg, MD). Cells are prepared and plated in DMEM/F12 medium (Life Technologies) containing 25mM HEPES buffer (no sodium bicarbonate), 10% fetal calf serum (FCS), penicillin/streptomycin (PS), and 2mM L-glutamine. Cells are plated

at a density of $4x10^4$ per well in a 96-well microtiter plate in a volume of 200ul per well. Cells are allowed to settle for approximately 30 minutes at room temperature and the plate is then placed in a 37°C humidified air incubator.

Parathyroid hormone induction of GFP-PTHR internalization. A 100uM stock of bovine parathyroid hormone (PTH), amino acids 1-34 (Bachem, King of Prussia, PA), is prepared using acidified water (pH 4-4.5). To induce internalization of the GFP-PTHR chimera, cells are stimulated by the addition of 50ul of 500 nM PTH to each well (diluted in DMEM/F12, 10% FCS, PS, 2mM L-glutamine). This volume is added to the 200ul of medium already in the well, yielding a final concentration of 100nM PTH. The plate is incubated at room temperature for two hours while covered with aluminum foil to protect the fluorophore from light. Following the two hour PTH stimulation, the media is decanted from the plate and the cells are fixed and the nuclei stained by the addition of 200ul of Hank's Balanced Salt Solution (HBSS) containing 3.7% formalin (Sigma) and 1ug/ml Hoechst 33342 (Molecular Probes, Eugene, Oregon). After a 10 minute incubation at room temperature, the solution is decanted from the plate, cells are washed by the addition of 200ul/well HBSS, and the plates are analyzed/stored with fresh HBSS (200ul/well).

Image acquisition and analysis. (See **Figure 26** for overview) After autofocusing 101 (**Figure 27**) on the Hoechst-labeled nuclei, an image of the nuclei 102 is acquired at 20x magnification. The nucleus image is segmented by thresholding 103, using a threshold value selected by the user or obtained by one of two other methods from which the user can select (isodata algorithm or peak interpolation). The total area in pixels of all the nuclei in the image is then computed as a single sum 104. An image of the GFP fluorescence is then acquired at 20x magnification 105. (**Figure 26**) The area of the plate imaged in this way is called a field.

Large artifacts are removed from the GFP image as follows 106. (**Figure 26**) The image is thresholded at a user-selected intensity value which is higher than the threshold used to detect valid objects later. All objects detected in the resulting image are labeled and their size (number of pixels) is measured. Any objects greater than a user-specified size are treated as artifacts. All such objects are copied and pasted onto a new blank image, the artifact image. The artifact image is dilated slightly to be sure that the artifacts will be completely

deleted. The artifact image is then subtracted from the original GFP image, yielding the intermediate image.

To remove variations in the background fluorescence, the intermediate image is subjected to a top hat transform 107. (**Figure 26**) This transform consists of (a) grayscale erosion (replacement of each pixel value by the minimum value in its neighborhood) (b) a grayscale dilation (replacement of each pixel value by the maximum value in its neighborhood) with the same size neighborhood as (a), producing a background image, and (c) subtraction of the resulting background image from the original input image to produce the object image, which contains small bright spots. The size of the neighborhood used for steps (a) and (b) above is selected to be slightly larger than all the objects of interest in the image. As a result, all such objects are absent from the background image after the erosion (a) and dilation (b). However, gradual variations in the background of the original image are retained in the background image. Therefore, the subtraction step (c) removes these variations in the background from the object image.

The object image is processed to determine which bright spots represent the internalized receptor in stimulated cells. This process uses a brightness threshold and a minimum size set by the user. The object image is thresholded at the brightness threshold to create the binary object mask 108. (**Figure 26**) The objects in the binary object mask are labeled and their sizes are measured in pixels. Those objects that meet or exceed the minimum size are valid spots 109; (**Figure 28**) the rest are ignored.

The following measurements are then determined for each valid spot. (**Figure 28**) The count of spots in the field is incremented 110. The number of pixels was previously counted. For each valid spot, the region with its label is extracted from the binary object mask to create the single-spot binary mask. The single-spot binary mask is applied to the original object image to get the grayscale spot image of the respective spot. The intensities of the pixels in the grayscale spot image are summed to get the aggregate intensity of the spot 111. Once all the spots have been processed, the sum of all of the areas of the valid spots are summed to get the aggregate spot area for the field 112. The aggregate intensity of the spots is totaled to get the aggregate spot intensity of the field. There are several statistics to choose from for the final score for the field (or well): (a) the number of valid spots; (b) the aggregate area of the valid spots; (c) the aggregate intensity of the valid spots; (d) the aggregate intensity of the valid spots divided by the total area of the nuclei. When more than

one field is analyzed within each well, the values for all the fields of the well are averaged together to get an aggregate statistic for the well 113. (**Figure 26**)

The following examples of determining receptor internalization using the above techniques illustrate the differences found between treated and untreated cells. The nuclei of unstimulated cells are labeled with the DNA-specific Hoechst stain and imaged with a near-UV fluorescence filter set. The same cells are imaged with a blue fluorescence filter set which shows the distribution of the GFP fluorescence. The nuclear mask is derived by applying a threshold to the nucleus-labeled image, and the background image is derived by the grayscale erosion and dilation of the GFP image, showing the variations in the background intensity. The object image is then derived by subtracting the background image from the GFP image, resulting in faint spots. The object mask is then derived by applying the threshold to the object image. Some faint spots are eliminated by the thresholding. Some others have fewer pixels over the threshold than the requirement for a valid spot. As a result, very few valid spots are found in the image of unstimulated cells. The spot count, aggregate spot areas, and aggregate spot brightness all have low values.

In a second example, the nuclei of stimulated cells are labeled with the DNA-specific Hoechst stain and imaged as in the preceding example. The nuclear mask is derived by the automated thresholding method, and the background image is derived by the grayscale erosion and dilation of the GFP image, showing the variations in the background intensity. The object image is derived by subtracting the background image from the GFP image, resulting in bright spots. The object mask is derived by applying the threshold to the object image. Many spots are seen in the object mask, and many of those spots have enough pixels over the threshold to meet the requirement for valid spots. The spot count, aggregate spot areas, and aggregate spot brightness all have high values. Results from experiments like these examples were shown previously in Figure 25.

Figure 29 shows a representative display of a PC screen showing data which was obtained by the methods described in the above examples. Each data point represents the Spot Count of a single well of the plate, calculated by summing together the Spot Counts of the fields of the well. The graph 300 shows individual curves, each representing a single row of the 96 well plate. The leftmost six points of each curve represent the Spot Counts of untreated wells, while the rightmost six points represent treated wells. The Spot Count feature ("obj count" in illustration) can be selected using the list 302. The numerical values for all the rows are shown in spreadsheet format 303. The graph 300 and spreadsheet 303 can

be printed, and the spreadsheet can be exported in a comma-separated format for input into a spreadsheet program such as Microsoft Excel ™.

Alternatively, the data can be displayed on a field by field basis (**Figure 30**). Each graph at the top 304, 305, and 306 can be set to plot any one of the computed statistics (averaged over the fields of the well) vs. the well number. The spreadsheet 307 shows the numerical data computed on a field by field basis. Selection of a line from the spreadsheet causes display of the corresponding Hoechst 308 and GFP 309 images to be displayed. The spreadsheet 305 can be printed or exported in an ASCII file format for input into a spreadsheet program such as Microsoft Excel ™.

The graph 304 shows the Spot Count vs. the well number. The Spot Count is the number of valid spots detected in the input GFP images. The invention provides a computer means for converting the digital signal from the camera into this parameter and for plotting the parameter vs. the well number.

The graph 305 shows the aggregate spot area ("total spot area" in illustration) vs. the well number. The aggregate spot area is the summed areas of all valid spots detected in the input GFP images. The invention provides a computer means for converting the digital signal from the camera into this parameter and for plotting this parameter vs. the well number.

The graph 306 shows the normalized spot intensity ratio ("Spot Inten Ratio x 100" in illustration) vs. the well number. The normalized spot intensity ratio is the summed intensities of all the pixels in the valid spots detected in the input GFP images, divided by the summed number of pixels in the nucleus masks in the corresponding Hoechst image. The invention provides a computer means for converting the digital signal from the camera into this parameter and for plotting the parameter vs. the well number.

Figure 25 is a graphical representation of data from validation runs of the PTHR internalization screen. The figure illustrates that the data for min. ("minimum response" = unstimulated) and max. ("maximum response" = stimulated) are consistent between different plates (the differences are not statistically significant), giving c.o.v.'s (coefficients of variance) within a consistent and acceptable range.

In a specific example of a high-content screen, four fields were acquired in each well. The Spot Count was summed across the fields of a well, and averaged among the similarly treated wells. The untreated half of the plate had a Spot Count of 69.3 ± 17.7 (mean ± Standard Deviation) times the untreated half of the plate, giving a Coefficient of Variation (COV, the Standard Deviation divided by the mean) of 26%. The values from the fields of

the treated half of the plate had a Spot Count of 404.2 ± 41.2, giving a COV of (10%). The mean Spot Count of the treated half was 5.83 times the mean Spot Count of the untreated half.

Example 5 Kinetic High Content Screen

Simply detecting the endpoint as internalized or not, may not be sufficient for defining the potency of a compound as a receptor agonist or antagonist. In another embodiment, the cells are treated with drug and data are collected at various timepoints following drug treatment in order to quantitate the kinetics of receptor internalization. These kinetic assays can be done on live cells as described above, or different wells of cells can be fixed at each of the various timepoints of interest following drug treatment. In either case, cells can be labeled using the reagents and methods described above. Such kinetic measurements would provide information not only about potency during the time course of measurement, but would also allow extrapolation of the data to much longer time periods.

In a preferred embodiment, kinetic measurements are first made in one channel of fluorescence in a high-throughput or ultra-high-throughput mode for a cellular response associated with receptor internalization. This response is less receptor specific than the internalization process itself and may include, but is not limited to, changes in Ca^{2+}, cAMP, or IP_3 concentrations, or activation of any of a variety of kinases. Wells exhibiting the desired output from this parameter are then analyzed in the HCS mode for highly detailed temporal and spatial information on a cell-by-cell basis.

The luminescence signals of live or fixed cells are analyzed using a cell scanning system, such as the cell scanning system of the present invention.

Example 6. Inserted Sequences and their Ligands for High-Content Screens Incorporating Dual-Labeled Receptors

In another embodiment, a membrane receptor is modified to contain specific peptide sequences fused to each end in order to distinctly label the extracellular and intracellular domains. A ratio of fluorescence intensity of the two labels is made in unstimulated and stimulated cells; since the amino terminus of the receptor is only available for labeling while the receptor is inserted in the plasma membrane, the ratio of the two labels in unpermeabilized cells can be used to measure the extent of internalization of the receptor. There is currently no known technology for simultaneously measuring the relative extracellular availability of external and internal domains of membrane receptors.

Appropriate cells are transiently or stably transfected with a DNA construct (either plasmid or viral based) that expresses the GPCR of interest fused to an epitope tag at its amino terminus and a molecular based chromophore at its carboxy terminus. Alternatively, the GPCR may be fused to an epitope tag at its carboxyl terminus and a molecular based chromophore at its amino terminus. The expression of the GPCR fusion may be constitutive or inducible.

Some examples of epitope tags include, but are not limited to, FLAG (Sigma Chemical, St. Louis, MO), myc (9E10) (Invitrogen, Carlsbad, CA), 6-His (Invitrogen; Novagen, Madison, WI), and HA (Boehringer Manheim Biochemicals). The expression the GPCR fusion may be constitutive or inducible.

Examples of useful molecular-based chromophores include, but are not limited to, GFP and any of its various mutants (Heim and Tsien (1996) Current Biology 6: 178-182; Zhang et al. (1996) Biochem. Biophys. Res. Comm. 227: 707-711). In addition, any of the luciferases and their mutants could also be used. The use of a luciferase as part of a chimeric target protein comprises a novel labeling technique since the examples of use of this molecular-based chromophore to date have included use as a reporter of gene activity (Yang et al. (1998) J. Biol. Chem. 273(17): 10763-10770; Peng et al. (1998) J. Biol. Chem. 273(27): 17286-17295; Baldari et al. (1998) Biologicals 26(1): 1-5)) and construction of biosensors (Campbell and Patel (1983) Biochem. J. 216: 185-194; Sala-Newby and Campbell (1992) FEBS Lett. 307: 241-244; Jenkins et al. (1990) Biochem. Soc. Trans. 18: 463-464) but not as a chimera for marking a particular protein target. Expression of the membrane protein-luminescent protein fusion may be constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone, steroid-responsive).

Alternatively, cells are transiently or stably transfected with a DNA construct (either plasmid or viral based) that expresses the membrane protein of interest fused to two distinct epitope tags, with one being fused to each end of the membrane protein.

The external availability of the inserted sequences depends on the internalization state of the receptor. That is, the ratio of the external availability of the inserted sequences provides a direct measure of the magnitude of receptor internalization. This is a high-content screen incorporating dual-labeled receptors. The external availability of the inserted sequences can be measured using a single approach or a combination of several approaches:

1. One or more of the inserted sequences can be epitopes for specific antibodies. Antibody binding to the epitope can be measured using histochemical, radioactive, or fluorescence methods. Possible epitopes include, but are not limited to, those shown in Table 1.

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TABLE 1. PEPTIDE EPITOPES AND THEIR CORRESPONDING ANTIBODIES

ANTIBODY	EPITOPE	SOURCE
FLAG	MDYKDDDDK	Sigma
Myc	EQKLISEEDL	Invitrogen, Boehringer-Mannheim Biochemical(BMB)
6-His	HHHHHH	Invitrogen, BMB, Berkley Antibody Company (BAbCO)
AU1	DTYRYI	BAbCO
AU5	TDFYLK	BAbCO
Glu-Glu	EEEEYMPME	BAbCO
HA	YPYDVPDYA	BMB, BAbCO
IRS	NPDSEIARYIRS	BAbCO
KT-3	KPPTPPPEPET	BAbCO
Protein C	EDQVDPRLIDGK	BMB
VSV-G	YTDIEMNRLGK	BMB
HSV	QPELAPEDPED	Novagen
T7	MASMTGGGQQMG	Novagen
V5	GKPIPNPLLGLDST	Invitrogen
Xpress™	DLYDDDDK	Invitrogen

2. The inserted sequences can code for fluorescent proteins. Besides the natural fluorophores of trp, tyr, and phe that exist in many proteins, other fluorescent protein sequences can be inserted. The GFP sequence or one of its mutant variants can be inserted into the sequence coding for the receptor. Sequences coding for luciferase and its mutant variants can also be inserted. Any peptide sequence that codes for or interacts with a fluorophore can be used in this method. The inserted sequences can be structured to express fluorescent proteins with different fluorescent properties such that fluorescent signals from each can be measured independently.

3. The inserted sequences can code for peptides that bind small (<1000 M_r) ligands with high affinity ($K_d < 10^{-9}$) and specificity. These small molecules then form a tight bridge to other molecules or macromolecules that can be luminescently or radioactively labeled. The inserted sequences can be structured to bind different bridging molecules that bind distinctly labeled molecules or macromolecules such that signals from each can be measured independently. For example, the peptide sequence –HHHHHH- will bind a metal ion (*e.g.*, Ni^{2+}, Cu^{2+}, *etc.*) that will form a tight bridge with a polydentate acetic acid moiety (*e.g.*, nitriloacetic acid). The acid moiety can be covalently linked to molecules that are luminescent, radioactive, or otherwise light absorbing. These molecules can be luminescent dyes or macromolecules such as proteins that contain a

luminescent or radioactive label. Other examples of inserted peptide sequences are such that they have a high affinity for other small molecules that include steroid hormones, vitamins, and carbohydrates that form a tight bridge to other molecules or macromolecules that can be luminescently or radioactively labeled.

Example 7. A Generalized Dual-labeled Receptor Internalization High-Content Screen

A modified G-protein coupled receptor (GPCR of known function or orphan) is transfected into human epithelial kidney cells (HEK 293) where its localization provides a measure of internalization from the plasma membrane. The modified GPCR contains an epitope (for example, FLAG) label at the N-terminus (extracellular) and a GFP-molecule at the C-terminus (intracellular). To measure GPCR internalization after ligand treatment, cells are fixed and labeled with Hoechst 33342 (a DNA-binding fluorescent dye) and a distinct luminescently labeled antibody against the epitope tag. A cell screening system, such as the cell screening system of the present invention, using ratio imaging, is used to calculate the internalization of the GPCR due to the loss of GPCR-epitope from the external side of the plasma membrane and an increase in GFP-only-labeled receptor within the cell. This approach to measuring ligand-induced receptor internalization is independent of the internalization mechanism so it is therefore applicable to a wide range of receptors of both known and unknown function.

Example 8 High-content screen of human glucocorticoid receptor translocation

One class of HCS involves the drug-induced dynamic redistribution of intracellular constituents. The human glucocorticoid receptor (hGR), a single "sensor" in the complex environmental response machinery of the cell, binds steroid molecules that have diffused into the cell. The ligand-receptor complex translocates to the nucleus where transcriptional activation occurs (Htun et al., *Proc. Natl. Acad. Sci.* 93:4845, 1996).

In general, hormone receptors are excellent drug targets because their activity lies at the apex of key intracellular signaling pathways. Therefore, a high-content screen of hGR translocation has distinct advantage over *in vitro* ligand-receptor binding assays. The availability of up to two more channels of fluorescence in the cell screening system of the present invention permits the screen to contain two additional parameters in parallel, such as other receptors, other distinct targets or other cellular processes.

Plasmid construct. A eukaryotic expression plasmid containing a coding sequence for a green fluorescent protein – human glucocorticoid receptor (GFP-hGR) chimera was prepared using GFP mutants (Palm et al., *Nat. Struct. Biol.* 4:361 (1997). The construct was used to transfect a human cervical carcinoma cell line (HeLa).

Cell preparation and transfection. HeLa cells (ATCC CCL-2) were trypsinized and plated using DMEM containing 5% charcoal/dextran-treated fetal bovine serum (FBS) (HyClone) and 1% penicillin-streptomycin (C-DMEM) 12-24 hours prior to transfection and incubated at 37°C and 5% CO_2 . Transfections were performed by calcium phosphate co-precipitation (Graham and Van der Eb, *Virology* 52:456, 1973; Sambrook et al., (1989). *Molecular Cloning: A Laboratory Manual*, Second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989) or with Lipofectamine (Life Technologies, Gaithersburg, MD). For the calcium phosphate transfections, the medium was replaced, prior to transfection, with DMEM containing 5% charcoal/dextran-treated FBS. Cells were incubated with the calcium phosphate-DNA precipitate for 4-5 hours at 37°C and 5% CO_2, washed 3-4 times with DMEM to remove the precipitate, followed by the addition of C-DMEM.

Lipofectamine transfections were performed in serum-free DMEM without antibiotics according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). Following a 2-3 hour incubation with the DNA-liposome complexes, the medium was removed and replaced with C-DMEM. All transfected cells in 96-well microtiter plates were incubated at 33°C and 5% CO_2 for 24-48 hours prior to drug treatment. Experiments were performed with the receptor expressed transiently in HeLa cells.

Dexamethasone induction of GFP-hGR translocation. To obtain receptor-ligand translocation kinetic data, nuclei of transfected cells were first labeled with 5 µg/ml Hoechst 33342 (Molecular Probes) in C-DMEM for 20 minutes at 33°C and 5% CO_2. Cells were washed once in Hank's Balanced Salt Solution (HBSS) followed by the addition of 100 nM dexamethasone in HBSS with 1% charcoal/dextran-treated FBS. To obtain fixed time point dexamethasone titration data, transfected HeLa cells were first washed with DMEM and then incubated at 33°C and 5% CO_2 for 1 h in the presence of 0 – 1000 nM dexamethasone in DMEM containing 1% charcoal/dextran-treated FBS. Cells were analyzed live or they were rinsed with HBSS, fixed for 15 min with 3.7% formaldehyde in HBSS, stained with Hoechst 33342, and washed before analysis. The intracellular GFP-hGR fluorescence signal was not diminished by this fixation procedure.

Image acquisition and analysis. Kinetic data were collected by acquiring fluorescence image pairs (GFP-hGR and Hoechst 33342-labeled nuclei) from fields of living cells at 1 min intervals for 30 min after the addition of dexamethasone. Likewise, image pairs were obtained from each well of the fixed time point screening plates 1 h after the addition of dexamethasone. In both cases, the image pairs obtained at each time point were used to define nuclear and cytoplasmic regions in each cell. Translocation of GFP-hGR was calculated by dividing the integrated fluorescence intensity of GFP-hGR in the nucleus by the integrated fluorescence intensity of the chimera in the cytoplasm or as a nuclear-cytoplasmic difference of GFP fluorescence. In the fixed time point screen this translocation ratio was calculated from data obtained from at least 200 cells at each concentration of dexamethasone tested. Drug-induced translocation of GFP-hGR from the cytoplasm to the nucleus was therefore correlated with an increase in the translocation ratio.

Results. Figure 20 schematically displays the drug-induced cytoplasm 253 to nucleus 252 translocation of the human glucocorticoid receptor. The upper pair of schematic diagrams depicts the localization of GFP-hGR within the cell before 250 (A) and after 251 (B) stimulation with dexamethasone. Under these experimental conditions, the drug induces a large portion of the cytoplasmic GFP-hGR to translocate into the nucleus. This redistribution is quantified by determining the integrated intensities ratio of the cytoplasmic and nuclear fluorescence in treated 255 and untreated 254 cells. The lower pair of fluorescence micrographs show the dynamic redistribution of GFP-hGR in a single cell, before 254 and after 255 treatment. The HCS is performed on wells containing hundreds to thousands of transfected cells and the translocation is quantified for each cell in the field exhibiting GFP fluorescence. Although the use of a stably transfected cell line would yield the most consistently labeled cells, the heterogeneous levels of GFP-hGR expression induced by transient transfection did not interfere with analysis by the cell screening system of the present invention.

To execute the screen, the cell screening system scans each well of the plate, images a population of cells in each, and analyzes cells individually. Here, two channels of fluorescence are used to define the cytoplasmic and nuclear distribution of the GFP-hGR within each cell. Depicted in Figure 21 is the graphical user interface of the cell screening system near the end of a GFP-hGR screen. The user interface depicts the parallel data collection and analysis capability of the system. The windows labeled "Nucleus" 261 and "GFP-hGR" 262 show the pair of fluorescence images being obtained and analyzed in a

single field. The window labeled "Color Overlay" 260 is formed by pseudocoloring the above images and merging them so the user can immediately identify cellular changes. Within the "Stored Object Regions" window 265, an image containing each analyzed cell and its neighbors is presented as it is archived. Furthermore, as the HCS data are being collected, they are analyzed, in this case for GFP-hGR translocation, and translated into an immediate "hit" response. The 96 well plate depicted in the lower window of the screen 267 shows which wells have met a set of user-defined screening criteria. For example, a white-colored well 269 indicates that the drug-induced translocation has exceeded a predetermined threshold value of 50%. On the other hand, a black-colored well 270 indicates that the drug being tested induced less than 10% translocation. Gray-colored wells 268 indicate "hits" where the translocation value fell between 10% and 50%. Row "E" on the 96 well plate being analyzed 266 shows a titration with a drug known to activate GFP-hGR translocation, dexamethasone. This example screen used only two fluorescence channels. Two additional channels (Channels 3 263 and 4 264) are available for parallel analysis of other specific targets, cell processes, or cytotoxicity to create multiple parameter screens.

There is a link between the image database and the information database that is a powerful tool during the validation process of new screens. At the completion of a screen, the user has total access to image and calculated data (Figure 22). The comprehensive data analysis package of the cell screening system allows the user to examine HCS data at multiple levels. Images 276 and detailed data in a spread sheet 279 for individual cells can be viewed separately, or summary data can be plotted. For example, the calculated results of a single parameter for each cell in a 96 well plate are shown in the panel labeled Graph 1 275. By selecting a single point in the graph, the user can display the entire data set for a particular cell that is recalled from an existing database. Shown here are the image pair 276 and detailed fluorescence and morphometric data from a single cell (Cell #118, gray line 277). The large graphical insert 278 shows the results of dexamethasone concentration on the translocation of GFP-hGR. Each point is the average of data from at least 200 cells. The calculated EC_{50} for dexamethasone in this assay is 2 nM.

A powerful aspect of HCS with the cell screening system is the capability of kinetic measurements using multicolor fluorescence and morphometric parameters in living cells. Temporal and spatial measurements can be made on single cells within a population of cells in a field. Figure 23 shows kinetic data for the dexamethasone-induced translocation of GFP-hGR in several cells within a single field. Human HeLa cells transfected with GFP-hGR

were treated with 100 nM dexamethasone and the translocation of GFP-hGR was measured over time in a population of single cells. The graph shows the response of transfected cells 285, 286, 287, and 288 and non-transfected cells 289. These data also illustrate the ability to analyze cells with different expression levels.

Example 9 High-content screen of drug-induced apoptosis

Apoptosis is a complex cellular program that involves myriad molecular events and pathways. To understand the mechanisms of drug action on this process, it is essential to measure as many of these events within cells as possible with temporal and spatial resolution. Therefore, an apoptosis screen that requires little cell sample preparation yet provides an automated readout of several apoptosis-related parameters would be ideal. A cell-based assay designed for the cell screening system has been used to simultaneously quantify several of the morphological, organellar, and macromolecular hallmarks of paclitaxel-induced apoptosis.

Cell preparation. The cells chosen for this study were mouse connective tissue fibroblasts (L-929; ATCC CCL-1) and a highly invasive glioblastoma cell line (SNB-19; ATCC CRL-2219) (Welch et al., *In Vitro Cell. Dev. Biol.* 31:610, 1995). The day before treatment with an apoptosis inducing drug, 3500 cells were placed into each well of a 96-well plate and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. The following day, the culture medium was removed from each well and replaced with fresh medium containing various concentrations of paclitaxel (0 – 50 μM) from a 20 mM stock made in DMSO. The maximal concentration of DMSO used in these experiments was 0.25%. The cells were then incubated for 26 h as above. At the end of the paclitaxel treatment period, each well received fresh medium containing 750 nM MitoTracker Red (Molecular Probes; Eugene, OR) and 3 μg/ml Hoechst 33342 DNA-binding dye (Molecular Probes) and was incubated as above for 20 min. Each well on the plate was then washed with HBSS and fixed with 3.7% formaldehyde in HBSS for 15 min at room temperature. The formaldehyde was washed out with HBSS and the cells were permeabilized for 90 s with 0.5% (v/v) Triton X-100, washed with HBSS, incubated with 2 U ml^{-1} Bodipy FL phallacidin (Molecular Probes) for 30 min, and washed with HBSS. The wells on the plate were then filled with 200 μl HBSS, sealed, and the plate stored at 4°C if necessary. The fluorescence signals from plates stored this way were stable for at least two weeks after preparation. As in the nuclear translocation assay, fluorescence reagents can be designed to convert this assay into a live cell high-content screen.

Image acquisition and analysis on the ArrayScan System. The fluorescence intensity of intracellular MitoTracker Red, Hoechst 33342, and Bodipy FL phallacidin was measured with the cell screening system as described *supra*. Morphometric data from each pair of images obtained from each well was also obtained to detect each object in the image field (*e.g.*, cells and nuclei), and to calculate its size, shape, and integrated intensity.

Calculations and output. A total of 50-250 cells were measured per image field. For each field of cells, the following calculations were performed: (1) The average nuclear area (μm^2) was calculated by dividing the total nuclear area in a field by the number of nuclei detected. (2) The average nuclear perimeter (μm) was calculated by dividing the sum of the perimeters of all nuclei in a field by the number of nuclei detected in that field. Highly convoluted apoptotic nuclei had the largest nuclear perimeter values. (3) The average nuclear brightness was calculated by dividing the integrated intensity of the entire field of nuclei by the number of nuclei in that field. An increase in nuclear brightness was correlated with increased DNA content. (4) The average cellular brightness was calculated by dividing the integrated intensity of an entire field of cells stained with MitoTracker dye by the number of nuclei in that field. Because the amount of MitoTracker dye that accumulates within the mitochondria is proportional to the mitochondrial potential, an increase in the average cell brightness is consistent with an increase in mitochondrial potential. (5) The average cellular brightness was also calculated by dividing the integrated intensity of an entire field of cells stained with Bodipy FL phallacidin dye by the number of nuclei in that field. Because the phallotoxins bind with high affinity to the polymerized form of actin, the amount of Bodipy FL phallacidin dye that accumulates within the cell is proportional to actin polymerization state. An increase in the average cell brightness is consistent with an increase in actin polymerization.

Results. Figure 24 (top panels) shows the changes paclitaxel induced in the nuclear morphology of L-929 cells. Increasing amounts of paclitaxel caused nuclei to enlarge and fragment 293, a hallmark of apoptosis. Quantitative analysis of these and other images obtained by the cell screening system is presented in the same figure. Each parameter measured showed that the L-929 cells 296 were less sensitive to low concentrations of paclitaxel than were SNB-19 cells 297. At higher concentrations though, the L-929 cells showed a response for each parameter measured. The multiparameter approach of this assay is useful in dissecting the mechanisms of drug action. For example, the area, brightness, and fragmentation of the nucleus 298 and actin polymerization values 294 reached a maximum

value when SNB-19 cells were treated with 10 nM paclitaxel (Figure 24; top and bottom graphs). However, mitochondrial potential 295 was minimal at the same concentration of paclitaxel (Figure 24; middle graph). The fact that all the parameters measured approached control levels at increasing paclitaxel concentrations (>10 nM) suggests that SNB-19 cells have low affinity drug metabolic or clearance pathways that are compensatory at sufficiently high levels of the drug. Contrasting the drug sensitivity of SNB-19 cells 297, L-929 showed a different response to paclitaxel 296. These fibroblastic cells showed a maximal response in many parameters at 5 μM paclitaxel, a 500-fold higher dose than SNB-19 cells. Furthermore, the L-929 cells did not show a sharp decrease in mitochondrial potential 295 at any of the paclitaxel concentrations tested. This result is consistent with the presence of unique apoptosis pathways between a normal and cancer cell line. Therefore, these results indicate that a relatively simple fluorescence labeling protocol can be coupled with the cell screening system of the present invention to produce a high-content screen of key events involved in programmed cell death.

Example 10. Protease induced translocation of a signaling enzyme containing a disease-associated sequence from cytoplasm to nucleus.

Plasmid construct. A eukaryotic expression plasmid containing a coding sequence for a green fluorescent protein – caspase (Cohen (1997), *Biochemical J.* 326:1-16; Liang et al. (1997), *J. of Molec. Biol.* 274:291-302) chimera is prepared using GFP mutants. The construct is used to transfect eukaryotic cells.

Cell preparation and transfection. Cells are trypsinized and plated 24 h prior to transfection and incubated at 37°C and 5% CO_2. Transfections are performed by methods including, but not limited to calcium phosphate coprecipitation or lipofection. Cells are incubated with the calcium phosphate-DNA precipitate for 4-5 hours at 37°C and 5% CO_2, washed 3-4 times with DMEM to remove the precipitate, followed by the addition of C-DMEM. Lipofectamine transfections are performed in serum-free DMEM without antibiotics according to the manufacturer's instructions. Following a 2-3 hour incubation with the DNA-liposome complexes, the medium is removed and replaced with C-DMEM.

Apopototic induction of Caspase-GFP translocation. To obtain Caspase-GFP translocation kinetic data, nuclei of transfected cells are first labeled with 5 μg/ml Hoechst 33342 (Molecular Probes) in C-DMEM for 20 minutes at 37°C and 5% CO_2. Cells are washed once in Hank's Balanced Salt Solution (HBSS) followed by the addition of

compounds that induce apoptosis. These compounds include, but are not limited to paclitaxel, staurosporine, ceramide, and tumor necrosis factor. To obtain fixed time point titration data, transfected cells are first washed with DMEM and then incubated at 37°C and 5% CO_2 for 1 h in the presence of 0 – 1000 nM compound in DMEM. Cells are analyzed live or they are rinsed with HBSS, fixed for 15 min with 3.7% formaldehyde in HBSS, stained with Hoechst 33342, and washed before analysis.

Image acquisition and analysis. Kinetic data are collected by acquiring fluorescence image pairs (Caspase-GFP and Hoechst 33342-labeled nuclei) from fields of living cells at 1 min intervals for 30 min after the addition of compound. Likewise, image pairs are obtained from each well of the fixed time point screening plates 1 h after the addition of compound. In both cases, the image pairs obtained at each time point are used to define nuclear and cytoplasmic regions in each cell. Translocation of Caspase-GFP is calculated by dividing the integrated fluorescence intensity of Caspase-GFP in the nucleus by the integrated fluorescence intensity of the chimera in the cytoplasm or as a nuclear-cytoplasmic difference of GFP fluorescence. In the fixed time point screen this translocation ratio is calculated from data obtained from at least 200 cells at each concentration of compound tested. Drug-induced translocation of Caspase-GFP from the cytoplasm to the nucleus is therefore correlated with an increase in the translocation ratio. Molecular interaction libraries including, but not limited to those comprising putative activators or inhibitors of apoptosis-activated enzymes are use to screen the indicator cell lines and identify a specific ligand for the DAS, and a pathway activated by compound activity.

Example 11. Cell State Identification

The specific association of labeled molecules with cells or cellular organelles can be used to characterize cells as being in a particular physiological state. Thus, in another embodiment, the present invention provides methods for characterizing the physiological state of individual cells within a population, comprising (a) providing an array of locations containing cells, wherein the cells possess a luminescently labeled cell identification reporter molecule and one or more luminescently labeled cell state reporter molecules, wherein emission from the cell identification and the cell state luminescently labeled reporter molecules are distinguishable; (b) imaging multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the cell identification and the cell state reporter molecules; (c) converting the luminescent signals into digital data; (d) using the digital data to create a mask of the cell identification reporter molecule and the cell state

reporter molecule; and (e) determining the intensity of the cell state reporter molecule mask that co-localizes with the cell identification reporter molecule mask, wherein if this intensity is above or below a certain threshold, the cell is characterized as being in a particular physiological state.

In a preferred embodiment, the cells are contacted with a test compound to determine the effect of the test compound on the physiological state of the cell. The present method can be used in either fixed cell, or live cell assays. In a further embodiment, the cells are contacted with a control compound known to modify the physiological state of the cells, and then contacted with the test compound to determine whether the test compound inhibited the control compound from modifying the physiological state of the cells. The contacting of the cells with the control compound can occur before, after, or simultaneously with the contacting of the cells with the test compound.

As used herein, the term "cell identification marker" refers to a marker that can be used to label all cells in a cell population of interest. In a preferred embodiment, the cell identification reporter molecule comprises a nuclear-specific stain. In alternative embodiments, the cell identification reporter molecule comprises a stain specific for bulk cytoplasm or the cell membrane of all cells, or for a specific cytoplasmic structure, including but not limited to the Golgi apparatus, the trans-Golgi network, endosomes, lysosomes, mitochondria, proteasomes, or endoplasmic reticulum; and cytoskeletal structures including microfilaments, and microtubules. Cell identification markers that are specific for structures that occur more than once per cell can be used so long as the various structures can be made to associate with each other, using image processing techniques such as dilation, blurring, etc., to form a single cell identification mask.

As used herein, the term "cell state marker" refers to a reporter molecule that can be used to identify cells in a particular physiological state, based on the presence in a cell of the target for the cell state reporter molecule. When multiple cell state reporter molecules are used, the emission from each luminescent cell state reporter molecule is distinguishable from the other(s). The method can analyze images from as many different cell state markers as permitted by the cell screening instrument.

As used herein, the phrase "the cells *possess* one or more luminescent reporter molecules" means that the luminescent reporter molecule may be expressed as a luminescent reporter molecule by the cells, added to the cells as a luminescent reporter molecule, or

luminescently labeled by contacting the cell with a luminescently labeled molecule, such as a dye or antibody, that binds to the reporter molecule.

As used herein, the "array" can be any substrate that allows for cell patterning and subsequent imaging, including but not limited to standard microplates, as well as microwell arrays (U.S. Patent No. 6,103,479).

The method generally involves using a fluorescent cell identification marker to identify all the cells in the population, and using a fluorescent cell state marker to determine whether an individual cell in the population is in a particular physiological state. This fluorescently labeled cell state marker has a different fluorescence spectrum than the cell identification marker, which enables those cells that are in the particular physiological state to be distinguished from the rest of the cells in the population. The fluorescence of the cells are then detected by an automated cell imaging instrument such as that described herein, and then an automated image processing method determines whether each cell in the population is in that particular state.

The automated image processing method first identifies and counts all the cells in the population, based on the presence of the fluorescent cell identification marker. This cell identification marker is used as a mask. The intensity of the fluorescent cell state marker that is co-localized with the cell identification mask area is then measured. Optionally, to reduce the effect of uneven illumination and to improve detection (1) a smoothing filter is applied to the cell identification and/or cell state image, and (2) background compensation is applied to all images prior to determining the cell identification and /or cell state mask. if this intensity is above or below a user-defined threshold, the cell is said to be in a particular physiological state. The mask area can be set as the same, smaller or larger than the area covered by the fluorescent cell identification marker.

The images containing the cell state marker may be more noisy that the cell identification image and can have non-compact areas for a single cell. Thus, the method optionally can further comprise (1) performing dilations or erosions on the cell state mask prior to determining its overlap with the cell identification mask; this step can fill in gaps (with dilations) or eliminate isolated small regions (with erosions); and/or (2) measuring the size (in pixels) of the overlap area between the cell identification mask and the cell state mask, and applying a threshold to that size.

The present method permits field-based or cell-based image acquisition and analysis. The method permits measuring and reporting of any number of parameters for each field

and/or well, including but not limited to the number of cells; the number of cells labeled with each cell state marker; the percentage of cells labeled with each cell state marker, and the normalized or average cell state marker luminescence intensities in the area defined by the nuclear mask. In addition, when multiple cell state markers are used, the method can measure the number and percentage of cells stained with one cell state marker but not the other, and the number and percentage of cells labeled with both cell state markers. Additional features that can be reported are the average and total areas of the cell identification and/or cell state masks.

Due to the availability of different fluorophores that span the visible and near-infra-red spectrum, several different fluorescent markers can be used to identify whether the individual cells in a population are in several different physiological states. The states can be mutually exclusive (e.g. the cell is alive or dead) or the cell can be in several different states at the same time. For example, in a brain cell population, different fluorescent markers could assess whether the cell (1) is a neuron, (2) is alive, and (3) expresses certain neuron-specific proteins. In this case, if a marker to identify all the cells in a population were also used, four different fluorophores would be needed to assess whether the cells are in these three different states. Cross-correlation analysis between the different states will give a more complete characterization of the different states of the individual cells in the population.

This type of cell population characterization can be used to gain information about the physiological state of cells in a wide variety of situations. Some examples of physiological states that can be quantitatively assayed by this approach include, but are not limited to, cell viability, pathogenic infection of cells, receptor internalization, cell type, foam cell formation, and adipogenesis.

To illustrate the wide applicability of the present invention, the following non-limiting examples are provided.

a. Cell Viability

Most compounds, beyond a certain concentration, are toxic to cells and will cause cell death. In trying to identify drug candidate lead compounds, it is important to determine the compound's concentration range that is non-toxic to cells. Another useful parameter is the compound's LD_{50} (50% lethal dose) which is the concentration where 50% of the cells in a population are dead.

Thus, the present methods can be used for automated cell-viability screens. In a preferred embodiment of the cell viability screen, the luminescent cell identification reporter molecule comprises a membrane permeable nucleic acid stain. In alternative embodiments, a first luminescent cell state reporter molecule comprises a plasma membrane permeable marker of live cell activity, or a membrane-impermeant nucleic acid stain that is specific for non-viable cells. In a preferred embodiment, the plasma membrane permeable marker of live cell activity comprises an esterase substrate, such as chloromethyl fluorescein diacetate (CMFDA).

In a further preferred embodiment, the cells are contacted with a second luminescently labeled cell state reporter molecule that is specific for either dead cells if the first luminescently labeled cell state reporter molecule is specific for live cells; or live cells if the first luminescently labeled cell state reporter molecule is specific for dead cells.

In a still further preferred embodiment, the cells are contacted with a test compound to determine the effect of the test compound on cell viability, wherein such effects include, but are not limited to, a dose response determination, time course of the response, or the compound's LD_{50}. Contacting of the cells with the test compound can occur either before, or simultaneously with, contacting of the cells with the stains in a fixed cell assay.

Thus, in one embodiment, the cell viability screen comprises providing an array of locations comprising multiple cells; wherein the cells possess a luminescently labeled cell identification reporter molecule that labels all cells and a first luminescently labeled cell state reporter molecule that is specific for either (a) live cells, or (b) dead cells; wherein emission from the luminescently labeled cell identification and first cell state reporter molecules are distinguishable; imaging multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the luminescently-labeled cell identification and first cell state reporter molecules; converting the luminescent signals into digital data; and utilizing the digital data to automatically make measurements, wherein the measurements are used to automatically calculate a percentage of cells that are viable. The cell viability assay is preferably a fixed-cell assay, but can also be performed as a live cell assay. In a preferred embodiment, the digital data are used to create a mask of the cell identification reporter molecule and the cell state reporter molecule, and detecting the intensity of the cell state reporter molecule mask that co-localizes with the cell identification reporter molecule mask.

By taking advantage of the different wavelengths available in the visible spectrum, these cell-viability screens can also be used in combination with high-content screens that

report other cellular phenomena, including but not limited to those described throughout the application. This allows the joint determination of whether a tested compound causes another specific cell physiological change, in addition to whether it is toxic to cells at the particular concentration used. Non-limiting examples of other screens that can be used in conjunction with the cell viability screen are nuclear fragmentation, mitochondrial mass potential, and lysosomal physiology screens.

The cell viability screens of the present invention consist of three parts:

1) Labeling the cells with fluorescent dyes that will indicate which cells are alive or dead;
2) Fluorescent imaging of the cells on a cell screening system, such as the ArrayScan®, which is disclosed in U.S. Patent Nos. 5,989,835 and 6,103,479; and
3) Image acquisition and analysis to identify the live and dead cells in the fluorescent image.

In a preferred embodiment, the method further comprises contacting the cells with a test compound to determine the effect of the test compound on the viability of the cell. In a further embodiment, the cells are contacted with a control compound known to modify the viability of the cells, and then contacted with the test compound to determine whether the test compound inhibited the control compound from modifying the viability of the cells. The contacting of the cells with the control compound can occur before, after, or simultaneously with the contacting of the cells with the test compound.

The basis of the screen is that when cells die (or are dying) their membranes become leaky and permeable to large macromolecules. This allows the entry of membrane-impermeant markers, including nucleic-acid dyes such as propidium iodide, and the loss of soluble cytoplasmic markers, such as esterases. Cytoplasmic esterase activity in live cells causes the retention and converts the non-fluorescent live-cell indicator chloromethyl fluorescein diacetate (CMFDA) into a fluorescent product. In the present assay, all the cells in the population are identified by Hoechst 33342 labeling. The dead cells are identified by the presence of nuclear propidium iodide ("PI") fluorescence in the red channel, and live cells are identified by cytoplasmic CMFDA fluorescence in the green channel. As used herein, "channel" refers to an optical component setting, such as a filter setting, to detect a specific wavelength of emitted fluorescence. When test or control compounds are used, the cells are treated with the test and/or control compounds, and then labeled with CMFDA and PI before fixation. This is followed by nuclei labeling with Hoechst, or another method for labeling all cells, and imaging on a cell screening system. The assay was developed and validated for HeLa and COS cells, and is widely applicable to different cell types.

Three specific cell viability assays have been developed that determine whether a cell is alive or dead:

1) Dead Cell Assay: The dead cells in the imaged field are fluorescently labeled with a membrane-impermeant nucleic acid stain and quantified;
2) Live Cell Assay: The live cells are fluorescently labeled with a plasma membrane permeable marker of live cell activity that comprises an esterase substrate, including but not limited to CMFDA; and
3) Combined Live/Dead assay: Both the live and dead cells in a population are identified by differently colored fluorescence markers, as in (1) and (2) above.

Identifying All The Cells In The Population:

In all three types of screens, a plasma-membrane permeable cell identification marker is used to identify all the cells in a population, such as a nuclear stain or a stain for a specific cytoplasmic structure. The present assay was designed to use fluorescent nucleic acid stain Hoechst 33342 (Molecular Probes; Eugene, OR), which passes through the plasma membrane of live or dead cells and labels the nuclei. It has a blue fluorescence emission, leaving the rest of the visible spectrum available for fluorescence from other parts of the cell-viability screen, as well as other high content screens. Other nucleic acid stains with different emission wavelengths that can pass through the plasma membrane of live and dead cells (such as the Molecular Probes series of SYTO® dyes) can be used in place of Hoechst 33342 to identify all the cells in a population. In this case, the blue wavelengths are available for other multiparameter HCS screens.

Dead Cell Assay:

The basis of the dead cell assay is to use membrane-impermeable cell state markers, such as fluorescent nucleic acid stains. These fluorophores can only stain the nuclei of dead cells whose plasma membranes have become leaky. This assay was developed using the dead cell stain PI, which fluoresces in the red channel and can be distinguished from Hoechst 33342's blue fluorescence. However, other membrane impermeable fluorescent markers for other cellular structures or organelles can be used, including but not limited to fluorescent antibodies or dyes specific for intracellular organelles such as the Golgi apparatus, the trans-Golgi network, endosomes, lysosomes, mitochondria, or endoplasmic reticulum; specific for cellular structures such as cytoskeletal elements including microfilaments, microtubules; or specific for other cellular structures, such as proteasomes or lipid droplets.

The number of Hoechst 33342 labeled nuclei gives the total number of cells in the population, and the number of nuclei labeled with PI gives the number of dead cells in the population. Since live cells' plasma membranes are impermeant to PI, their nuclei will only have Hoechst 33342 fluorescence.

Other dead cell nucleic acid stains that can be used with appropriate optimization include, but are not limited to, those in Table 2:

Table 2. Dead Cell Stains

Nucleic Acid Stain	Catalog Number in Molecular Probes' Handbook (6th Edition)	Excitation & Emission Maxima (nm/nm)
YOYO®-1	Y-3601	491/509
SYTOX®	S-7020	504/523
POPO™-3	P-3584	534/570
Propidium Iodide	P-3566	535/617
Ethidium Homodimer 2	E-3599	535/624
BOBO™-3	B-3586	570/604
YOYO®-3	Y-3606	612/631
TOTO™-3	T-3604	642/660

Live Cell Assay:

Our approach for a live cell stain is to introduce a fluorescent cell state marker into the cell which then is or becomes membrane-impermeant and cannot leave the cell. Thus, the cells that are alive will be fluorescent due to the marker. When the cell membrane becomes compromised (leaky) then the live-cell, cell state marker will leave the cell, causing a reduction in the cell's fluorescent intensity. This strategy requires introduction of an appropriate fluorescent cell state marker into the cell.

Non-limiting examples of methods for introducing such fluorescent cell state markers into cells include:

(1) Use of a marker that is membrane permeable by virtue of being hydrophobic, and which is retained in the cell because the hydrophobic portion of the molecule is hidden or removed after entry into the cell. For example, CMFDA contains two acetate groups that allow it to cross the plasma membrane into the cell; subsequent cleavage of the acetate groups by cellular esterases cause the compound to be retained in the cell. The acetoxymethyl ester form of calcein is introduced into cells via a similar technique. This technique requires that the marker be membrane permeable, and to subsequently become impermeable after

entry into the cell. The remaining techniques do not require that the marker be membrane permeable to enter the cell, but do require that the marker not be membrane permeable after entry into the cells, or that it be minimally membrane permeable, as a slow leakage out of the cell is acceptable.

(2) Use of an osmotic shock procedure to introduce an impermeant fluorescent marker into cells. In this method, the fluorescent marker is in the extracellular liquid, and is taken up by fluid phase pinocytosis. An osmotic shock to the cells (i.e. briefly putting the cells in a hypo-osmolar solution such as water) causes the internalized vesicles containing the marker to burst, releasing its contents (the fluorescent marker) into the cytoplasm.

(3) Use of a bead loading, scrape loading, or related mechanical technique to introduce an impermeant fluorescent marker into cells. In this method, the fluorescent marker is in the extracellular liquid. Mechanical agitation to the cells is applied (such as rolling small beads over them) and this causes small tears in the cell membranes making them temporarily leaky. This allows the fluorescent marker to be introduced into the cell. The cell membrane then heals, and thus the marker is trapped in the cell.

(4) Use of electroporation to introduce an impermeant fluorescent marker into cells. In this method the fluorescent marker is in the extracellular liquid. The brief electrical voltage pulse is applied to the media that causes the cell membranes to become temporarily leaky, allowing the fluorescent marker to be introduced into the cell. The cell membrane then heals, and thus the marker is trapped in the cell.

A wide range of fluorescent cell state markers can be introduced into cells via these techniques, including but not limited to fluorescent antibodies, fluorescent dextrans or beads, and fluorescent dyes without hydrophobic groups, and can thus be used as live cell markers in the present assay.

In a preferred embodiment, a plasma membrane permeable cell state marker of live cell activity is used, such as fluorogenic esterase substrates, which are plasma membrane permeable fluorophore-ester conjugates. Intracellular esterases cleave the esters, trapping the fluorophore in the cytoplasm of the cell; its fluorescence identifies the cell as alive. Dead cells have compromised membranes, and both the intracellular live-cell fluorophore and intracellular esterases leave the cell. Thus, dead cells are identified by having reduced or no fluorescence.

The examples described below use the fluorogenic esterase substrate CMFDA, which is not fluorescent and, due to its two diacetate esters, can diffuse into the cytoplasm of live

cells. Cellular esterases cleave the acetates, making the CMFDA fluorescent with green emission. In addition, the chloromethyl group reacts with intracellular thiols, resulting in a cell-impermeant fluorescent dye-thioether adduct that can optionally be fixed with aldehyde fixatives, if one chooses not to perform live cell screening. Thus, live cells are those that have green fluorescence. If the cell is dead and the cellular esterases have leaked out, the cell will not become fluorescent.

Although this assay has been developed and optimized for CMFDA, other live cell stains can be used that are similar to CMFDA but that have different spectra. An example is CMTMR (chloromethyl tetramethylrhodamine) which fluoresces in the red. A different type of fluorogenic esterase substrate to identify live cells is calcein, which is the live cell stain in a commercially available LIVE/DEAD® kit (Molecular Probes). The acetoxymethylester form of calcein (calcein AM) can diffuse through the plasma membrane of live cells. Cytoplasmic esterases cleave the acetoxymethylester trapping calcein inside the cell. Calcein has a green fluorescence emission, and live cells are identified as those cells that have green fluorescence. If the cell is damaged or dead, its membrane becomes permeant and the intracellular calcein leaks out. Thus, dead cells exhibit reduced or no fluorescence over background. This assay is performed on live cells, as the membranes of fixed cells become leaky. A drawback to using calcein is that some cells may actively pump the dye into mitochondria or out of the cell. The advantage to using CMFDA (or CMTMR) over calcein is that the samples can be fixed and viewed later, thus obviating the need to image the cells under conditions where they need to be kept alive.

Combined Live and Dead Cell Assay:

The above screens can be combined to create a joint live and dead cell screen. For example, the blue fluorescence of Hoechst 33342 would be used to identify all the cells in the population, PI's red fluorescence would identify those cells which are dead, and CMFDA's green fluorescence would identify the cells which are alive. This combined screen for live and dead cells provides a more robust measure of cell viability than individually screening for live or dead cells. Although this assay was developed and optimized for PI and CMFDA, Table 3 lists some other examples of dye combinations that can be used for this combined Live and Dead Cell assay.

Table 3: Examples of Dye Combinations for the Combined Live and Dead Cell Assay:

Identification of All Cells	Identification of Dead Cells	Identification of Live Cells
Hoechst 33342	YOYO®-1	CMTMR
Hoechst 33342	SYTOX®	CMTMR
Hoechst 33342	POPO™-3	CMFDA
Hoechst 33342	Propidium Iodide	CMFDA
Hoechst 33342	Ethidium Homodimer 2	CMFDA
Hoechst 33342	BOBO™-3	CMFDA
Hoechst 33342	YOYO®-3	CMFDA
Hoechst 33342	TOTO®-3	CMFDA or CMTMR

Demonstration Killing Agent:

The protocol for these assays is given in the next section. For these assays, Tween 20 (Sigma Chemical Co., St. Louis, MO) was chosen as the demonstration cell-killing agent since at intermediate concentrations (2.0% - 0.2%), some of the cells were alive and some were dead. This allowed the determination of a Tween 20 dose response curve. However, at the intermediate concentrations, cell loss from the plates was seen, resulting in higher variability in the percentage of cells that were alive or dead. Although this cell loss did not occur at high Tween 20 concentrations, it must be realized that cell loss may occur when other killing agents are used. To minimize cell loss, the cells for these assays were plated on poly-L-lysine (PLL) (ICN Biochemicals) coated microwell plates. In addition to reporting the percentage of live or dead cells, the method also reports the number of nuclei (i.e. number of cells), so that cell loss can be monitored.

Detailed embodiment of cell treatment

1. Plate 200 µl of cells into each well of a 96 well PLL treated plate at a density of 7.5 X 10^3 cells/well 18-24 hours in advance of experiments.
2. Remove 180 µl medium from wells by aspiration and add Eagle's Minimal Essential Medium (MEM) 2% FCS (live control) or the same medium containing 4% Tween 20 (dead control) to the appropriate wells. Solutions should be pre-warmed to 37°C.
3. Incubate for 30 minutes at 37°C, 5% CO_2.
4. Aspirate 200 µl of fluid from wells and add 200 µl of MEM without FCS pre-warmed to 37°C to wash.
5. Add 200 µl of diluted dye(s) prewarmed to 37°C. There are three dye options:
    i. live assay: CMFDA only
    ii. dead assay: PI only

iii. live/dead assay: CMFDA and PI together

6. Incubate for 15 minutes at 37°C, 5% CO_2.

7. Aspirate 200 µl of fluid from wells and add 200 µl of MEM without FCS to wash.

8. Incubate for 5 minutes at 37°C, 5% CO_2.

9. Aspirate 200 µl of fluid from wells, add 200 µl of fixative (warmed to 37°C); and add Hoechst 33342.

10. Incubate at room temperature for 10 minutes.

11. Aspirate 200 µl of fluid from wells, add 200 µl of PBS (room temperature) to wash.

12. Aspirate 200 µl of fluid from wells, add 200 µl of PBS (room temperature) to wash. This wash is allowed to remain in place.

13. Seal plate using plate seal, store at 4°C.

The only plate layout requirements are that:

1. Nuclei be present in those wells where tag-point auto-focusing occurs; otherwise mis-focusing will occur; and
2. Positive and Negative control wells must exist for at least one plate per stack or batch of plates; this allows the determination of the fixed exposure time and channel 2 (and 3) fixed thresholds (see below).

Once the plates are ready, they are scanned on the cell screening system. In running stacks of plates, the exposure time was determined manually on specific positive and negative control wells on the first plate. Examples of positive control wells are:

-Dead Cell Assay: Treat with 4% Tween20 for 30 minutes at 37°C to kill cells. Their nuclei will be have red fluorescence from PI.

-Live Cell Assay: Treat with media for 30 minutes at 37°C to keep cells alive. They will have green fluorescence from CMFDA.

Examples of negative control wells are:

-Dead Cell Assay: Treat with media for 30 minutes at 37°C to keep cells alive. Their nuclei will not have PI fluorescence.

-Live Cell Assay: Treat with 4% Tween20 for 30 minutes at 37°C to kill all cells. They will not have CMFDA fluorescence.

The exposure time is determined so that the control wells give the correct results (i.e. close to 100% for the positive control wells and near 0% for the negative control wells). Once the exposure time has been determined for both the PI and CMFDA channels, the whole stack of 96-well plates is run with these times.

The method used to scan the plates and analyze the results was the "Field Based Cell State" method, which identifies objects in a primary channel (cell identification markers) and then identifies whether there is fluorescence above a threshold in these objects in the other fluorescent channels. The Field-Based Cell State method can analyze as many secondary channels (i.e.: cell state markers) as the cell screening system provides, in addition to the primary channel, and reports on a field by field basis the number of objects detected in the secondary channels and what percentage they are of the objects detected in the primary channel. For example, in the combined live/dead assay, Hoechst labeled nuclei are the objects detected in the primary channel (blue), and the percentage of nuclei that have PI or CMFDA (i.e. that are dead or alive, respectively) are reported in the secondary channels (red and green, respectively). The response variable (raw data) typically reported is the percentage of cells containing the dye (i.e. the percentage of dead or alive cells).

In addition to this method, a Cell-Based Cell State method has also been developed that can be used for these cell viability assays. The Cell-Based Cell State method can detect and analyze as many secondary channels as the cell screening system provides, and reports, on a cell by cell basis, whether the cell is alive or dead.

The methods measure and report the following features for each field, and/or well: the number of cells (by counting nuclei); the number of cells stained with each live and/or dead cell dye; the percentage of cells stained with each live and/or dead cell dye. In addition, when a live and a dead cell dye are used, the method measures the number and percentage of cells stained with each dye but not the other, and the number and percentage of cells stained with both dyes. Additional features reported are the average and total nuclear areas, and the normalized dye 2 and dye 3 intensities in the area defined by the nuclear mask.

Two Channel Version

This method can measure the percentage of cells in a field that are in a particular viability state. A *Positive State* means that the cell is brightly fluorescent, and a *Negative State* means that the cell has hardly any fluorescence. When a dead-cell stain is used, a *Positive State* occurs for every dead cell, and a *Negative State* for each live cell. Similarly,

when a live-cell stain is used, a *Positive State* occurs for every live cell, and a *Negative State* for each dead cell. Two images are captured per field (or per cell, if using the Cell Based Cell State method): one at a specific fluorescence wavelength where only the nuclei are stained, and the other image at another wavelength where cells are stained to detect their state by either a live-cell stain, or a dead-cell stain. The method first makes a mask of the nuclei. Optionally, to reduce the effect of uneven illumination and to improve detection (1) a smoothing filter is applied to the nucleus channel image, and (2) background compensation is applied to all images. In the other channel, it is determined if the cell is in the *Positive State* by whether there is fluorescence in the area delineated by the nuclear mask. The number of cells in a *Positive State* is compared to the total number of all cells (i.e. nuclei) to obtain the percentage of *Positive State* cells in a field.

A threshold is applied to the nuclear image, producing the nucleus mask. For segmenting nucleus images, various intensity threshold methods can be used, including but not limited to the isodata method or the entropy method. A manual fixed threshold can also be used. For the embodiments described herein, the isodata method was used. The image where all the nuclei are stained has a very high S/N ratio, and the count of nuclei is a very stable and reliable measure of the total number of cells present in the image. The images of nuclei are very consistent, and have intensity distributions that (1) are strongly bimodal due to both the background and the nuclear labeling, and (2) generate nucleus areas that are compact (i.e. give single 8-connected components for a single nucleus). Therefore, a simple 8-connected components labeling is sufficient to count the number of nuclei. 8-connected components is a method of determining which of the pixels that have intensities above an intensity threshold are connected and belong to the same object. In an 8-connected components scheme, if any of the 8 pixels surrounding the pixel of interest have an intensity above the intensity threshold, it is identified as part of the same object as the central pixel. The user can optionally specify morphologic dilations or erosions to the nucleus mask.

Next, an intensity threshold is applied to the live and/or dead cell image to distinguish cells in the *Positive State* from those in the *Negative State*. In the image where cells in a *Positive State* are stained, cells that are in a *Negative State* are still slightly fluorescent, and their pixels are brighter than background pixels. Therefore, a threshold is applied to detect pixels that are in a *Positive State*. The *Positive State* mask is created by means of a simple threshold (selection of pixels above *Positive State detection threshold*). Fixed thresholding is the preferred embodiment, although other automatic thresholding methods can be used that

properly assign objects found in negative control wells to the Negative State, and objects found in positive control wells to the Positive State. The user predetermines this threshold by experimenting with typical plates. The user can optionally background compensate the *Positive State* image by subtraction of local average.

The next step is to identify the cells to which the threshold-selected *Positive State* pixels belong. The images containing dead and live cells are usually more noisy that the nucleus-stained image and can have non-compact areas for a single cell. Thus a simple 8-connected components labeling may be insufficient to identify cells in the *Positive State*.

Two steps have been combined to solve this problem: (1) morphological operations (dilations or erosions) on the channel's *Positive State* pixel mask (the binary image formed by thresholding) prior to inspecting the masks for overlap with the nuclear mask; this step can fill in gaps (with dilations) or eliminate isolated small regions (with erosions); and (2) measuring the size (in pixels) of the overlap area between the nucleus of the cell and the stained area of the non-nucleus channel, and applying a threshold to that size. The user controls two parameters, one for each of these steps. For the first, a small integer is selected. A positive integer specifies the number of dilations applied to the thresholded non-nucleus channel; a negative integer specifies that erosions (numbering the absolute value of the negative number) be applied instead. Zero specifies that no morphological operations be applied. The second step requires that the user specify a minimum number of pixels to coincide in both the cytoplasmic and nuclear masks, for the cell to be in the *Positive State*. The selected area is logically ANDed with each separate 8-connected component of the nucleus mask. The area of the resulting object is compared with an area (i.e. pixel number) threshold (rejection threshold), and if larger, the cell is counted as *Positive State*. The number and percentage of cells which are in the *Positive State* are calculated.

The method reports the total number of cells, total nuclear area for all cells and/or *Positive State* cells only, average nuclear area for all cells and/or *Positive State* cells only, total number of *Positive State* cells, the percentage of the total cells which are in the *Positive State* for each field and/or well, the nuclear area per field or well, the average nuclear area per cell, and the normalized masked intensity of the *Positive State* fluorophore.

Three Channel Version

This method measures the percentages of dead and live cells in a field by capturing three images for each field: an image where only the nuclei are stained, an image where only dead cells are stained, and an image where only live cells are stained.

This method separately computes and stores the percentage of live cells from the nucleus image and the live-stained channel; and the percentage of dead cells from the nucleus image and the dead-stained channel. Each non-nucleus channel is treated exactly like the one non-nucleus channel in the two channel version described above. In addition, it does a computation of the number of ambiguous cells which are stained on both the live-stained and dead-stained channel. Therefore, in addition to the number (and percentage) of cells which are live-stained, the number (and percentage) of cells which are dead-stained, it also reports the number (and percentage) of cells which are live-stained but not dead-stained, the number (and percentage) of cells which are dead-stained but not live-stained, and the number (and percentage) of cells which are both live- and dead-stained.

In a preferred embodiment, the exposure time and/or thresholds are changed (or checked) whenever a stack of plates is run, as discussed above. Additionally, if the three channel version of the assay is run, the thresholds must be set so that the ambiguous cells (those which are in the *Positive State* with both the live cell and dead cell stains) are also at a minimum.

Two strategies can be used to optimize the thresholds for the secondary channel or channels:

1. For the easier, less sensitive method, a min/max plate is scanned where half the wells contain all dead cells and the other half all live cells. Repeated disk-based scans should be performed, using the saved images, and successively changing the selected thresholds, until acceptable rates of error are achieved. The threshold selected this way should be useful for the entire batch of plates.

2. If the parameters need to be more carefully determined so they are sensitive to lower thresholds of *Postive* or *Negative State* cells, it will be useful to prepare a plate (preferably with each batch of plates) which has a titration curve of a toxic agent (e.g. Tween20) across at least one row. There should be control wells which receive no toxic agent, and the concentrations should range from a sub-threshold concentration to a uniformly lethal concentration. The treated row or rows should then be scanned, using a guess as to the appropriate thresholds. The images should be saved during this scan. Then, repeated disk-based scans should be performed, using the saved images,

to successively refine the selected thresholds, until acceptable rates of error are achieved. The threshold selected this way should be useful for the entire batch of plates.

Validation Data

Validation runs were done for the three Cell Viability assays, and following are summary results from three validation reports:

1. *Dead Cell Assay Validation Report:* HeLa cell were used in a fixed cell assay. The assay was used to determine the percentage of cells with nuclear Propidium Iodide staining (i.e. % of cells that are dead), using Tween 20 as a control compound. The results indicated a very high signal to noise ratio, and minimal variability from well to well, plate to plate, or assay to assay.

2. *Live Cell Assay Validation Report:* HeLa cell were used in a fixed cell assay. The assay was used to determine the percentage of cells with CMFDA staining (i.e. % of cells that are alive), using Tween 20 as a control compound. The results indicated a very high signal to noise ratio (slightly lower than for the dead cell assay), and minimal variability from well to well, plate to plate, or assay to assay.

3. *Combined Live/Dead Cell Assay Validation Report:* HeLa cell were used in a fixed cell assay. The assay was used to determine the percentage of cells with CMFDA and nuclear PI staining (i.e. % of cells that are alive and dead, respectively), using Tween 20 as a control compound. The results indicated a very high signal to noise ratio (slightly lower than for the dead cell assay), and minimal variability from well to well, plate to plate, or assay to assay.

b. Quantifying the amount of pathogen in a cell population

The development of drugs that affect the infection of cells by pathogens, such as viruses, bacteria or parasites can also be aided by use of the present methods. Such a drug-discovery process would be facilitated by the ability to easily and rapidly quantify the amount of infection of a population of cells by a certain concentration of the pathogen (e.g. viral titer). The present methods can be used to quantify the amount of pathogen infection in a cell population. The following example is for detecting viral infection, but similar strategies can be used to screen for bacterial and parasitic infection of cells.

The basis of the screen is to have the virus (or other pathogen) identified by a fluorescent cell state marker. Then the cells are imaged in an automated manner, and the amount of the pathogen's fluorescence per cell is automatically quantified, where all cells are identified by a cell identification reporter, such as a nuclear dye or cytoplasmic dye, as discussed above.

Examples of fluorescent cell state markers specific for the virus (or pathogen) include, but are not limited to, (1) antibodies against viral proteins, which are used to label the virus by immunofluorescence after infection; (2) Organic fluorophores that are used to pre-label the virus prior to infection of test cells; or (3) Chimeras between viral proteins and biological fluorophores, such as the autofluorescent protein GFP (green fluorescent protein).

The cell population is infected by the virus, and depending on which of the above fluorescent methods for virus identification is used, the virus is fluorescently labeled either pre- or post-infection. The cells are then fixed and labeled with a fluorescent nucleic acid cell identification marker, to identify all the cells in the population. The cell identification marker fluorescence is chosen to have a different spectrum than that of the fluorescently labeled cell state marker. This allows one to quantify which cells contain the virus, as well as the degree of the viral infection in the cell, and over the population of cells. In a preferred embodiment, Hoechst 33342, which fluoresces in the blue, is used as the cell identification marker. However, many other cell identification markers can be used, as discussed above The fluorescently labeled cells are then imaged on a cell screening system in the same manner as for other High Content Screens. The viral infection in each cell and over the population of cells in each imaged field is then quantified by the cell state method described above, where the amount of cell state fluorescence intensity that is co-localized with the cell identification mask is measured. If the co-localized cell state fluorescence intensity exceeds a user-set threshold, the cell is identified as being infected by the virus. In addition, the amount of fluorescence intensity in each cell can be measured to quantify the degree of infection. In this case, the degree of infection is a further qualification of the infected cell state. This method has been used to quantify HeLa cell infection by commercially available adenovirus that express GFP (QBiogene, Carlsbad, CA).

A similar strategy can be used to quantify the bacterial infection of cells. The bacteria are identified by a fluorescent cell state marker either by (1) immunofluorescence, (2) pre-labeling it with an organic fluorophore, or (3) by having it express an autofluorescent protein such as GFP. The cells infected by the fluorescently labeled cell state marker would then be

imaged on the cell screening system, identified, and counted as described above for virally infected cells. Wiater et al (Infect. Immun., 66(9):4450-4460) have used fluorescein labelled Legionella pneumophilla to quantify its infection. Live bacteria can be stained by the various fluorescent live bacteria labeling kits produced by Molecular Probes Inc. (Eugene, OR). Other references deal with pathogenic bacteria expressing GFP (Myobacterium avium: Microb. Pathog., 22(4):193-198, 1997; Yersinia and Salmonella: Gene, 173:47-52, 1996).

c. Quantifying Receptor Internalization.

When cell surface receptors are internalized, they enter and traffic through a system of intracellular compartments collectively known as endosomes. Certain receptors such as some G-protein coupled receptors (GPCR) traffic through an endosome known as the endocytic recycling compartment. Other receptors, such as the epidermal growth factor receptor traffic through an endosome known as the late endosome and then to the lysosome. There are different fluorescent markers for the different types of endosome and the lysosome. These markers can be used as cell identification masks to quantify whether receptor internalization and trafficking to particular endosomes has occurred. Examples of fluorescent endosome specific markers include fluorescent transferrin for the endocytic recycling compartment and fluorescent dextrans or fluorescent low density lipoprotein (such as diI-LDL) for the late endosome. Receptor internalization using these fluorescent markers can be quantified using the same methodic strategy as used to quantify cell viability. In addition, the endosomal markers can also be used to identify and count the cells.

d. Identifying a specific cell type in a mixed cell population.

In a heterogeneous population of cells, particular types of cells can be identified. For example, in a population of brain cells, specific fluorescent markers can be used to identify the different cells such as neurons, astroglia and microglia. In this case, fluorescently labeled cell state markers include, but are not limited to, antibodies against neurofilaments or βIII-tubulin, which are neuron-specific, to identify cells as neurons; antibodies against Glial Fibriliary Acidic Protein (GFAP) would bind to a protein specific to astroglia, and uptake of fluorescent acetylated low density lipoprotein is carried out specifically by microglia. All three markers could be labeled with fluorophores with different spectra enabling the simultaneous separation of all three cell types in the population.

In another example, in a population of blood cells, fluorescently labeled antibodies against specific antigens can be used as cell state markers to identify the different cells via indirect immunofluorescence. A third example is where all the cells in a population are of a similar type, but express different amounts of a particular cellular constituent such as a protein. If the cellular constituent can be fluorescently labeled, the fluorescence intensity of individual cells can be quantified to identify only those cells that express a certain amount of the constituent. In these examples, the cell states are the particular cell types or the range of the fluorescently identified constituent they contain. The cell identifying marker in this case can be a nuclear stain such as Hoechst 33342.

e. Quantifying cholesterol uptake by macrophages and the formation of foam cells.

Foam cells are macrophages that have taken up cholesterol from lipoproteins, and then have stored them as cholesteryl esters in lipid droplets. The formation of foam cells is the early critical event in the development of an atherosclerotic plaque. This aspect of the invention is an assay to detect and quantify the formation of foam cells by the presence of esterified cholesterol and unesterified cholesterol in macrophages. Macrophages (or macrophage-like cells such as J774 cells) can be fed atherogenic lipoproteins, including but not limited to VLDL (very low density lipoprotein), oxidized-LDL (low density lipoprotein) and acetylated-LDL, to induce the storage of cholesteryl esters in fat droplets and thus cause the cell's transformation into a foam cell.

Cholesteryl ester (CE)-loaded macrophages, (also known as foam cells) are a prominent feature of atherosclerotic plaques, and the formation of foam cells is the early critical event in the development of an atherosclerotic plaque. Foam cells are formed when macrophages take up cholesterol from atherogenic lipoproteins or other cholesterol-rich particles, esterify them with the enzyme ACAT (acyl:coenzyme A : cholesterol acyltransferase), and then store them in lipid droplets. The main effect of cholesterol in heart disease is in the formation of foam cells. Thus, agents that block or impede the formation of foam cells will be useful in the treatment and prevention of atherosclerosis, and foam cell formation and physiology is a major focus of research and interest by scientists interested in atherosclerosis.

This cell state method can be used to assay the formation of foam cells by the presence of esterified cholesterol and unesterified cholesterol in macrophages. In this assay, macrophages (or macrophage-like cells such as J774 cells) are fed atherogenic lipoproteins,

and then are treated with the fluorescent probes filipin, Nile Red, and CMFDA (chloromethyl fluorescein diacetate). CMFDA, which fluoresces in the green, is used to identify all the cells in the population and their areas. The antibiotic filipin fluoresces when it is bound to free, unesterified cholesterol in membranes (excitation and emission maxima of 357 and 480 nm, respectively), and the phenoxazine dye Nile Red fluoresces when it associates with neutral lipids such as cholesteryl esters (excitation and emission maxima of 552 and 636 nm, respectively). Thus, within each cell (cell area identified by CMFDA), the presence of unesterified cholesterol is indicated by filipin fluorescence, and the presence of esterified cholesterol is indicated by Nile Red fluorescence. This quantifies the degree of accumulation of cholesteryl esters, and thus the degree of foam cell formation.

f. Quantifying adipogenesis.

The cell state method can also be used to create a cellular domain based on dilation or erosion of the nuclear mask to include the perinuclear region of the cell where a majority of the lipid droplets accumulate. The fluorescent intensity of various cell state probes of lipid formation or accumulation or both, in this domain are measured and calculated. These measurements can be applied to different cell types such as SNB19, 3T3L1, pre-adipocytes, and adipocytes. Cell state probes that can be used for these measurements include, but are not limited to, probes that co-localize with intracellular lipid, fluorescently labeled lipids, or fluorescently labeled lipid precursors such as fatty acids. Nile Red can be also be used to detect lipid and fatty acid uptake by cells. The same method as used for cell viability can be used to do the quantification.

The present invention further comprises computer readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute the cell state methods disclosed herein, wherein the cell screening system comprises an optical system with a stage adapted for holding a plate containing cells, a digital camera, a device for directing light emitted from the cells to the digital camera, and a computer for receiving and processing the digital data from the digital camera.

The present invention further comprises kits for measuring cell viability comprising:

(a) a membrane permeable nucleic acid stain;

(b) a plasma membrane permeable marker of live cell activity;

(c) a membrane-impermeant nucleic acid stain; and

(d) instructions for using the kit to determine cell viability according to the methods described herein.

Example 12. Neurite Outgrowth

A major interest for drug discovery is the identification of compounds that affect the growth of neurites from neurons. Drugs that promote nerve growth will be of use for treating a wide variety of diseases and trauma that result in neuropathy and nerve injury, including but not limited to spinal cord injury, neuropathy resulting from diseases such as diabetes and stroke, Parkinson's disease, and other forms of dementia including Alzheimer's disease.

Thus, in another aspect, the present invention provides automated methods, kits, and computer readable media for analyzing neurite outgrowth. The methods of this embodiment comprise

-providing an array of locations comprising cells, wherein the cells possess at least a first luminescently labeled reporter molecule that reports on cell number, and at least a second luminescently labeled reporter molecule that reports on neurite outgrowth, wherein the cells comprise neurons;

-imaging or scanning multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the first and second luminescently-labeled reporter molecule;

-converting the luminescent signals into digital data; and

-utilizing the digital data to automatically make measurements, wherein the measurements are used to automatically calculate changes in the distribution, environment or activity of the first and second luminescently labeled reporter molecules on or within the cells, wherein the calculated changes provide a measure of neurite outgrowth.

As used herein, the term "neurons" or "neuronal cells" includes any cell population that includes neurons of any type, including, but not limited to, primary cultures of brain cells that contain neurons, isolated cell cultures comprising primary neuronal cells, neuronal precursor cells, tissue culture cells that are used as models of neurons (such as PC12 cells, which are a neoplastic neuronal cell line cloned from rat pheochromocytoma), or mixtures thereof.

As used herein, the term "neurite" refers to any processes and/or structures that grow from a neuron's cell body including but not limited to axons, dendrites, neurites, intermediate segments, terminal segments, filopodia and growth cones.

As used herein, the phrase "neurite outgrowth" includes positive neurite outgrowth, neurite outgrowth inhibition, neurite outgrowth degradation, and other changes in neurite morphology.

As used herein, the phrase "the cells possess one or more luminescent reporter molecules" means that the luminescent reporter molecule may be expressed as a luminescent reporter molecule by the cells, added to the cells as a luminescent reporter molecule, or luminescently labeled by contacting the cell with a luminescently labeled molecule that binds to the reporter molecule, such as a dye or antibody, that binds to the reporter molecule. The luminescent reporter molecule can be expressed or added to the cell either before, simultaneously with, or after treatment with the test substance.

In another embodiment, the method further comprises contacting the neurons with a test compound, and wherein the calculated changes indicate whether the test compound has modified neurite outgrowth in the neurons. If a mixed cell culture is used, and the nuclei of the other cells in the mixed culture are to be identified, such as the astrocytes, oligodendrocytes, or microglia, then fluorescent probes that are specific to those cell types and are labeled with a different fluorophore are used, and sufficient images per field (i.e. more than two) are acquired to identify astrocytes, oligodendrocytes, or microglia. This embodiment of the invention can be used to discover compounds that affect (positively or negatively) neurite outgrowth from neuronal cells, as well to identify conditions that are toxic to neurons and affect their neurites' morphology, including without limitation neurite length, number, and branching. For such neurotoxicity studies, the method would comprise identifying compounds that degrade neurites, or identifying test compounds that inhibit the activity of known neurotoxins.

In a preferred embodiment, the first luminescently labeled reporter molecule comprises a DNA binding compound. In a further preferred embodiment, the second luminescently labeled reporter molecule comprises a compound that selectively detects a cell component selected from the group consisting of cytoplasm, membrane, and cellular proteins. In a further embodiment, the second luminescently labeled reporter molecule is neuron-specific. In another embodiment, the cells comprise at least a third luminescently labeled reporter molecule that is neuron-specific, or specific to other cell types of interest, including but not limited to microglia, oligodendrocytes, and astrocytes.

In another embodiment, the method further comprises contacting the cells with a control compound known to modify neurite outgrowth, and utilizing the calculated changes to

determine whether the test stimulus inhibited the control compound from modifying neurite outgrowth in the neurons. Alternatively, no test stimulus is added, and the measurements and calculated changes are made after removal of the control compound, to determine the effects of such removal on neurite outgrowth.

In a further embodiment, sub-regions of the array of locations are sampled multiple times at intervals to provide kinetic measurement changes in the distribution, environment or activity of the luminescent reporter molecules on or within the cells

In addition, other high content or high throughput assays, including without limitation those described throughout the application, can be used in combination with the present assay, to measure the physiological state of the same neurons upon compound treatment. Preferred assays for use in a multiparametric assay with the present method are cell viability assays, apoptosis assays, and G-protein coupled receptor (GPCR) and other receptor internalization assays.

This aspect of the invention provides a way to automatically scan arrays of cell populations treated with different compounds and automatically quantify the neurite outgrowth of the neuronal cells both collectively and individually. The neurons do not have to be isolated from a mixture of different cell types or different neuronal cell types to be used in this embodiment, and thus the method can be applied to primary brain cultures.

The present invention further provides computer readable storage media comprising a program containing a set of instructions for causing a cell screening system to execute the methods of this aspect of the invention, wherein the cell screening system comprises an optical system with a stage adapted for holding a plate containing cells, a means for moving the stage or the optical system, a digital camera, a means for directing light emitted from the cells to the digital camera, and a computer means for receiving and processing the digital data from the digital camera. In a preferred embodiment, the cell screening system is that disclosed above.

The invention further provides kits for analyzing neurite outgrowth, or for identifying compounds that modify neurite outgrowth, comprising at least one neuron-specific luminescent reporter molecule; at least one nucleus-specific luminescent reporter molecule; and instructions for using the neuron-specific luminescent reporter molecule and the nucleus-specific luminescent reporter molecule to analyze neurite outgrowth, or to identify compounds that modify neurite outgrowth.

Identification of Neurons

In one embodiment, all cells in the sample are labeled with a luminescent reporter molecule marker to identify their locations. Once the cell locations are identified, a cell count can be made. Typically, the nucleic acid dye Hoechst 33342 is used as a luminescent reporter molecule to identify the nuclei of all the cells. However, other nuclear labels can also be used. Nucleic acid fluorescent stains are of two kinds: those that can cross the plasma membrane of live cells, and those that are membrane impermeant. Examples of membrane permeant nucleic acid stains include DAPI, dihydroethidium, hexidium iodide, Hoechst 33258, and the SYTO® dye series (Molecular Probes). To label nuclei with membrane-impermeant dyes, the plasma membrane has to be permeabilized. Examples of membrane-impermeant nucleic acid dyes include cyanine nucleic acid labels such as TOTO®, YOYO®, BOBO™, POPO™, TO-PRO®, YO-PRO®, BO-PRO™ and PO-PRO™ (Molecular Probes), ethidium analogs such as ethidium-acridine heterodimer, ethidium bromide, ethidium diazide and ethidium homodimers 1 and 2, propidium iodide, and the green nucleic acid stain SYTOX® (Molecular Probes). In addition, other components of the cells, such as the cytoplasm, can be labeled to identify all of the cells in the culture if the neurons are sparsely plated. In a preferred embodiment, a nuclear label is used. Examples of some cytoplasmic stains are given below.

If the sample consists of a mixture of brain cells (including cell types other than neurons), the luminescently labeled nuclei that belong to neurons are identified. The neurons are distinguished from the other cells by a neuronal specific luminescent reporter molecule with luminescence of a different wavelength from the nuclei marker and any other cell markers or other luminescent reporter molecules that are being used. The nuclei that coincide with the neuron-specific marker are identified as those nuclei in the mixed cell population that belong to neurons. There are many different neuron-specific labeling markers that can be used. Some examples of neuron specific labeling strategies include, but are not limited to: indirect immunofluorescence against neurofilaments, indirect immunofluorescence against βIII-tubulin, and indirect immunofluorescence against neurotrophic factors such as the ciliary neurotrophic factor (CNTF), all being neuron-specific antigens and proteins.

The cells are luminescently labeled so all of their processes can be visualized, whether the culture consists only of neurons or neuronal-like cells, or neurons in a mixed cell culture. There are several targets on neurons that can be luminescently labeled to allow visualization of the processes:

(1) Cytoplasmic Staining: The cytoplasm can be stained with any standard cytoplasmic stain. Examples of such stains are CMFDA (chloromethyl fluorescein diacetate), or CMTMR (chloromethyl tetramethylrhodamine) (Molecular Probes). Alternatively, the cells can be engineered to express an autofluorescent protein such as Green Fluorescent Protein (GFP). The expressed GFP in the cytoplasm will allow the neuron's processes to be visualized.

(2) Membrane Staining: The membrane stain can either be a standard lipid dye such as dil (dioctadecylindocarbocyanine) (Molecular Probes), or can be a fluorescently labeled protein that is on the cell's membrane. To fluorescently label proteins, one can use either immunofluorescence against cell surface proteins (using standard immunofluorescent staining techniques) or a fluorescent ligand that binds a membrane protein. This strategy can serve a dual purpose in that, in addition to identifying the neuron shape and processes, it can also be used to specifically and selectively identify neurons from a mixed brain culture. Examples of neuron specific markers that are on the membrane are the various neurotrophic factors. For example, indirect immunofluorescence against the ciliary neurotrophic factor CNTF on the surface of neurons can delineate the architecture of the neuron.

(3) Staining of Cellular Proteins: Certain cytoplasmic stains label cellular proteins, some of which are specific to neurons. This category includes cytoskeletal proteins that help delineate neurons. Example of this include, but are not limited to: indirect immunofluorescence against neurofilaments, or against βIII-tubulin, both which are neuron-specific cytoskeletal proteins.

(4) A combination of all of these staining strategies can be used to better identify the neuronal processes and outgrowing neurites.

Identification of compounds that stimulate neurite outgrowth

Cells are plated onto a substrate, which can be made of any optically clear material, including but not limited to glass, plastic, or silicon wafer, such as a conventional light microscope coverslip. In certain situations, a plastic substrate is sufficient for good attachment of the cells. However, for some cell types, the substrate needs to be coated with specific extracellular matrices for good attachment and growth. For example, PC12 cells need to be grown on a collagen substrate. The compound(s) to be tested are then added to the cells. After the appropriate amount of time the neuronal cells are luminescently labeled (if they were not previously labeled) and then images are acquired and analyzed automatically to

quantify neurite outgrowth, as described below. In some experiments done with PC12 cells treated with Nerve Growth Factor (NGF), the cells were luminescently labeled, imaged, and analyzed two to seven days after NGF treatment.

Identification of compounds that inhibit neurite outgrowth

The neuronal cells are first plated onto a substrate as above. The cells are treated with the compound to be tested and with a control compound (such as Nerve Growth Factor (NGF)) that is known to stimulate neurite outgrowth, wherein treatment with the control compound is done either before, after, or simultaneously with test compound treatment. After an appropriate time period, images are acquired and analyzed automatically to quantify neurite outgrowth, as described below.

Identification of conditions that are toxic to neurons and neurites

The neuronal cells are first plated onto a substrate as above, and treated to allow neurite outgrowth. For example, the cells could be contacted with NGF, as described above. After neurite outgrowth occurs, the cells are treated with the condition to be tested for toxicity towards neurons and neurites. Examples of such conditions could be addition of a concentration range of a potentially toxic compound, alteration of a physical parameter critical to the cells' growth, or in some cases, withdrawal of the factor that stimulates neurite outgrowth, such as NGF. After an appropriate time period images are acquired and analyzed automatically, as described below, to quantify the neurite outgrowth.

Image Acquisition and Analysis

When neuronal cells are sparse or do not have a large degree of neurite outgrowth, individual cells can be easily identified. However, as neurites start to grow and the cells start putting out numerous processes, these processes may intersect and the neuronal cells become part of a large cluster of cells. Thus, the entire cell cluster becomes a single connected luminescent entity. The different processes and structures that grow from a neuron's cell body include axons, dendrites, neurites, intermediate segments, terminal segments, filopodia and growth cones. For image acquisition and analysis, all of the processes and structures are classified into two groups: (1) the cell body (also known as soma), and (2) the neurites. The cell body is the central part of the neuron that contains the nucleus and has a roughly compact, round morphology. All of the outgrowths and processes emerging from the cell body are classified as neurites. A neurite may branch, intersect other neurites or have smaller processes growing from it, all of which are considered as part of their parent neurite for the purpose of image analyses. Thus, a neurite has one origin, which is in the cell body, but may

have multiple end points if it branches. The results obtained from applying the present method allow the user to define and classify the neurites according to their classification guidelines. For example, in one publication, axons are defined as the longest continuous neurite from the cell body, neurite segments between 0.7 μm and 5.1 μm in length are defined as filopodia, and those longer than 5.1 μm are called neurites if emerging from the cell body or terminal segments if an end is attached to a neurite (Ramakers et al, 1998, Developmental Brain Research, 108:205-216.

The neurite outgrowth methods of the present invention perform the following types of analyses:

a. Identify the cells' nuclei. If the sample is a mixed culture of cells, it identifies the nuclei which belong to neurons. The nuclei are used to identify and keep count of the neurons, and also to determine the number of cell bodies in a cluster of neurons;

b. Identify the degree of neurite outgrowth in the well and for the individual cell clusters (if the cells are sparse or neurite outgrowth is limited, the cell clusters would only consist of one cell). This is achieved by measuring the morphology of the neuronal cells (or cell clusters), which includes the cell body and the neurites extending from them;

c. Measure specific properties and morphological features of the neurites such as their lengths, number, and branch points;

d. Measure which and how many of the neurons can be considered positive for neurite outgrowth; and/or

e. Combine the analysis with other HCS analyses on the same cells or cell clusters. Examples of other HCS assays that can be applied include, but are not limited to assays for cell viability, apoptosis, or GPCR and other receptor internalization.

For example, in one embodiment of the method, the following features of the cells, neurons and neurites are measured and reported:

Parameter	Units	Description
Neurite Outgrowth Index	%	Percentage of neurons that are positive for neurite outgrowth (i.e. percentage of neurons whose summed neurite lengths are greater than a user entered minimum length threshold).
Degree of Outgrowth	Number	The neuron's form factor is used as a measure of the degree of neurite outgrowth. The form factor is the square of the cell's perimeter divided by 4π times its area. It is 1 for a circle, a little larger than 1 for cells without outgrowth, and much larger for cells with significant outgrowth and branching. This parameter is influenced by the number of neurites, their lengths, as well as their branching. The reported parameter is the mean form factor for all identified neurons and unresolved neuronal clusters.
Total Cells Counted	Number	Total number of cells determined from the nuclear stain (such as Hoechst 33342)
Number of Neurons	Number	Number of neurons. A cell is identified as a neuron if the intensity of the neuronal stain colocalized with a dilated nuclear mask is greater than a user entered minimum intensity threshold.
Number of Positive Neurons	Number	Number of positive neurons. Positive neurons are neurons whose summed neurite lengths are greater than a user entered minimum length threshold.
Number of Neurites per Positive Neuron	Number	Number of neurites from positive neurons normalized by the number of positive neurons.
Neurite Length per Positive Neuron	μm	Neurite length per positive neuron. Sum of neurite lengths from positive neurons normalized by the number of positive neurons.
Neurite Length per Neurite	μm	Neurite length per neurite. Sum of neurite lengths from positive neurons normalized by the number of neurites from positive neurons

TABLE 4: List Of Output Features Reported By The Current Version Of The Neurite Outgrowth Method.

In addition, the above features can be combined (such as to normalize one feature with the other, or to correlate two or more features) to be reported as new features.

A preferred method to quantify neurite outgrowth and measure these features is described below. As used therein, the following terms have the given meaning:

"Image" refers to a display of pixels that have intensities.

"Pixel" refers to an (x,y) coordinate location within the array, along with the associated intensity value.

"Binary Image" refers to an image in which each pixel has an intensity of either 0 or 1. This is usually derived from an image whose pixels have a full intensity range. Binarization assigns those pixels with an intensity above a threshold to have an intensity of 1 in the binary image. The pixels that have intensities less than or equal to the threshold have intensity 0 in the binary image. The pixels "contained in" a binary image are considered merely to be the

pixels that have value 1. The binarized image can also be used as a mask to be applied to other images to measure the intensities of the pixels that are colocalized with the binary masked structures.

"Thresholding" refers to the process of selecting those pixels of an image whose intensity lie above a value termed a threshold. The result of thresholding is stored within a binary image wherein pixels above the threshold have value 1 and the others have value 0.

"Adaptive Thresholding" refers to the process of automatically selecting and applying a suitable threshold value for an image by considering the brightness distribution present in the image. A variety of different methods of selecting the threshold are known; the one used in this assay is known as the "isodata method", but other autothresholding schemes can be used.

Connected Component: Any pixel (whose location is not along the image boundary) has 8 neighboring pixels (4 pixels with which it shares a side, and 4 others with which it shares a corner). A pixel is said to be *8-connected* to each of its 8 neighboring pixels. "8-connected components" is a method of determining which of the pixels that have intensities above an intensity threshold are connected and belong to the same object. In an 8-connected component scheme, if any of the 8 pixels surrounding the pixel of interest have intensities above the intensity threshold, it is identified as part of the same object as the central pixel. Consider the pixels which have intensity 1 in a binary image. These pixels may be divided into separate groups where the separate groups satisfy certain properties: 1) no group contains a pixel that is 8-connected to any pixel of any other group. 2) any two pixels within a group contain a path connecting them that passes through only pixels of that group. The groups satisfying these properties are termed *connected components* of the image.

The "Form-Factor" can be used as a quantitative measure of neurite outgrowth. It consists of the square of the image object's (e.g. cell or cell cluster) perimeter divided by four times π times the area of the object. (i.e. FF = perimeter^2 / (4π Area))

If no neurite outgrowth has occurred and the neuronal cell's shape is similar to a circle, the Form Factor will be close to 1. As neurite outgrowth occurs and the cell or cell cluster becomes more branched, the value of this FF measure increases. The average FF over the entire imaged field can be computed to give the degree of neurite outgrowth over the whole well. In addition, the degree of neurite outgrowth for individual neurons or neuronal-cell clusters can also be determined by their individual FF.

Background Compensation: In order to avoid sensitivity from uneven fluorescence distribution, a background compensation filter can be applied to the image. This stage removes low spatial frequency variations from the image. One strategy of doing this is to subtract the background intensity from a neighborhood around each pixel. In order to estimate the background intensity in the neighborhood of a pixel, we average the intensity within a square region centered upon the pixel. Since we do not want to use very bright pixels to form this estimate (very bright pixels are clearly foreground pixels and should not be included in an estimate of the background), we include only pixels lying below some intensity threshold to form the average. Having obtained the background intensity estimate, we subtract this intensity from the pixel intensity. The result, when this operation is performed over the entire image, is a background compensated image.

Branch Identification: A branch is a point when a neurite growing from the main cell body splits into more than one (usually two) neurite segments growing from the neurites. The branch-point or triple-point is the junction where a single branch splits into two or multiple branches. The image is analyzed to find and count branch points.

Image Acquisition

a. Preferred embodiment (See Figure 31A-B)

Inputs to the Method:

Two images are provided as input to the method: a nuclear channel image and a neurite channel image. In the nuclear channel image, the cells' nuclei are labeled with Hoechst 33342 or some other fluorescent or luminescent nuclear stain. In the neurite channel image, the neuronal cell body, and its attached neurites are fluorescently or luminescently labeled.

Initialization Phase:

The initialization phase commences with an optional background compensation on both the nucleus and neuron images. The background compensation stage is applied in order to reduce the effect of uneven illumination and to improve detection.

A binary image is generated for both the nuclear channel image and the neurite channel image by application of a threshold; auto-thresholding is the preferred method as it does not require user input.

Nuclear Channel:

A binary image is generated from the nuclear channel image by the application of an auto-threshold. One connected component is present in this kernel image per neuronal nucleus or nuclear clump. The location coordinate of each nucleus is determined by first applying the binary kernel image as a mask over the background-compensated nuclear image, and then using a peak-detection routine to select the pixel which has the peak maximum intensity. This pixel is tagged as the position coordinate for each nucleus.

Neurite Channel:

A binary reservoir image is generated from the neurite channel image by the application of an auto-threshold. The pixels in this reservoir image will generally be a superset of the pixels in the nuclear kernel image.

Identification of Cell Bodies

The neurons consist of cell bodies with neurites extending from them. Each cell body contains one nucleus, and the cell body covers a larger area than the nucleus. Before starting to quantify neurite outgrowth, the cell bodies, which are the source of the neurites, need to be identified. Thus, a series of dilations are performed from each nuclei's peak pixel until the connected components of the kernel image (which correspond to neuronal nuclei) expand to fill out the associated neuronal cell bodies. The dilations performed are termed *conditional dilations*; each time the dilation is applied, a layer of one pixel is added to the kernel on the condition that the pixels in that layer are present in the neuron reservoir image. This means that the increase in area due to the dilation has still not extended beyond the cell body's boundaries. During each dilation, the numbers *nfront* and *nadded* are measured for each connected component in the kernel image. *Nfront* is the number of pixels that would be added to the connected component by a simple (unconditional) dilation, which is just dilation by an additional pixel. *Nfront* can be thought of as the new perimeter, measured in number of pixels, of the object due to the latest dilation. *Nadded* is the number of pixels that are actually added by the conditional dilation – conditional because after a one pixel dilation, only those pixels in the new perimeter which are positive in the reservoir image are counted. Thus, *nadded* is the number of pixels in the new perimeter which has an intensity of 1 in the binary reservoir image. If the ratio *nadded/nfront* is computed to be less than some user-defined

number threshold in the course of a dilation (we find that a range from 0.05 to 0.3 empirically works with our test images), no more dilations are performed on that connected component. This means that the extent of the cell body has been reached, and no more dilations are required. The additional pixels in subsequent dilations that are positive in the binary reservoir image belong to actual neurites growing out from the cell body. The extent of the cell body can be reported as the cell body area. When all connected components have reached this stage (i.e. all the individual nuclei have been processed to this stage), then the next step of the method is initiated.

At this point, the kernel image contains one connected component (i.e. one entity) for each neuronal cell body.

Iteration Phase:

The next step is to identify the neurites extending from each cell body. For this, one conditional dilation is performed on the kernel image in order to identify each *neurite* stub. The term *dilation image* describes the image containing only the positive binary pixels added by a dilation. Each connected component in the dilation image is termed a *node*. Each node is used to initialize a one-node *neurite* data structure. A neurite data structure is intended to represent the physical neurite as it extends outward from the neuronal cell body, potentially containing multiple branches and potentially joining with other neurites.

Next, conditional dilations are successively performed upon the kernel image until no further pixels from the reservoir image remain that are contiguous to pixels of the kernel image. In the dilation image produced by each such dilation, the set of nodes is computed. Each node represents either the continuation of a neurite object, a branching of a neurite object, or the joining of two neurite objects. An association is formed between a node and an existing neurite object if one or more pixels of a node are adjacent to the pixels of the neurite object. If a node is associated with more than one neurite object, then it represents a join point. If multiple nodes are associated with a neurite object, then it represents a branch point. If a node is associated with just one neurite object and that neurite object is associated only with the said node, then the node is an extension of the neurite object. The extension, branch or join is recorded. In the case of joins, the neurite objects involved are merged.

The entire neurite is identified by linking together its set of connected nodes, and then the neurite's length is measured. Length threshold criteria may be applied to classify the different neurites. One application of such criteria would be to reject neurites that are too

short. Each neurite origin is associated with the neuron it originated from. One way of doing this is to link the neurite's origin node with either the nearest cell body or nuclear peak. In certain cell types (e.g. PC12 cells), the neurons form clusters and only a subset of cells within the cluster extend neurites. This association of neurites with their originating neurons identifies the neurites and their originating cells within a cluster of cells.

Output Features:

A variety of different quantities can be measured by this method. First, the number of cells and the number of neurons can be measured and reported. For each neurite, the total length (measured as the sum of the lengths of all its branches) and number of branches can be measured. For each nucleus, the number of neurites that emerge from it can be measured. For each cluster, the form-factor ($perimeter^2 \div (4\pi \times area)$) is measured and reported as the degree of neurite outgrowth. In addition, the neurite lengths from each nuclei are summed, and if greater than a length threshold, the nuclei are identified as being positive for neurite outgrowth. These measures can be combined in different ways to give the output features reported in **Table 2.**

Validation of Preferred embodiment:

1. Measurement of Neurite Outgrowth in PC6-3 cells

PC6-3 cells (a sub-clone of PC12 cells) were grown on 96-well microplates whose wells had been coated with collagen. The wells contained different concentrations of NGF (nerve growth factor) (0-1000 ng/ml) to stimulate neurite outgrowth. A control population did not contain NGF. After two days, the cells were fixed and indirect immunofluorescence was performed against βIII-tubulin, using a rabbit anti-βIII-tubulin primary antibody and an ALEXAFLUOR™ 488 conjugated goat anti-rabbit secondary antibody (Molecular Probes). The cells were then fixed in 3.7% formaldehyde for 20 minutes, and the fixative solution also contained 10 μg/ml Hoechst 33342 to label their nuclei. The cells were imaged on the cell screening system of the present invention and then analyzed with a prototype method described above. Results given below are for the form-factor and mean neurite length as a function of NGF concentration:

NGF Concentration (ng/ml)	Mean Neurite Length (μm)	Mean Form Factor
0	3.8	2.5

62.5	27.8	16.3
250	47.6	41.5
1000	64.4	51.7

2. Measurement of Dopamine Toxicity to Neurites from PC12 Cells

PC12 cells were grown for 7 days in the presence of 1 μg/ml NGF on 96 well microplates with collagen-IV coated wells. Varying concentrations of dopamine were added for 3 hours before the cells were fixed in 3.7% formaldehyde for 20 minutes; the fixative solution also contained 10 μg/ml Hoechst 33342. The cells were stained by indirect immunofluorescence using a rabbit primary antibody against βIII-tubulin, and an ALEXAFLUOR™ 488 conjugated goat anti-rabbit secondary antibody. The cells were imaged on the cell screening system of the present invention, and then analyzed with the prototype method described above. Results given below are for the Neurite Outgrowth Index (see Table 2) as a function of dopamine concentration. Each data point is the mean result from 8 wells, and error bars are the standard deviations. The IC_{50} (50% inhibitory concentration) for dopamine toxicity to neurites from this data is 0.46 mM.

Dopamine Concentration (mM)	Neurite Outgrowth Index (%) (mean +/- standard deviation)
0.0	46 +/- 5
0.05	43 +/- 4
0.1	37 +/- 3
0.2	37 +/- 3
0.4	25 +/- 3
0.8	17 +/- 2
1.6	11 +/- 2

b. Alternative image acquisition embodiment (See Figures 32 and 33A-B)

In an alternative embodiment, the method measures the percentage of cells in a field that are in a particular state, in this case, those that are neurons in a culture containing a mixed population of cell types. A Positive State means that the cell is brightly fluorescent, and a Negative State means that the cell has little or no fluorescence. When a neuron-specific reporter molecule is used, a Positive State occurs for every neuronal cell, and a Negative State

for all other cells. To identify neurons, two images are captured per field as discussed above. The number of cells in a Positive State is compared to the total number of all cells to obtain the percentage of Positive State cells in a field.

Furthermore, a mixture of different types of neurons can be assayed for neurite outgrowth. Each neuronal sub-population to be analyzed is identified by a distinct reporter molecule. Such a method can be used, for example, to distinguish GABAnergic neurons from cholinergic neurons in a mixed population, by immunofluorescence against the specific neurotransmitters' receptor.

This alternative embodiment can be summarized as follows:

Detection Threshold Computation

Two control wells are used: a sample where all the cells are in the Positive State, and a sample where all cells are in the Negative State. The Positive State cells are brightly labeled and the Negative State cells are not. In order to improve cell segmentation, all images can be background compensated by subtracting the local average intensity over a user determined area. For the negative control, a threshold is set to minimize the variance of the intensity distributions of the non-cell background and the cells. For the positive control, a threshold is set to minimize the variance of the intensity distributions of the non-cell background and the cells. The Positive State detection threshold is set as a weighted sum of the thresholds computed from the control images.

Detection and Counting of Nuclei (Hoechst Labeled)

The nuclear image is background compensated by subtraction of local average. A threshold is applied to the image. The image has a bimodal intensity distribution due to the dim pixels from the non-cell background and the brighter pixels associated with cells. The threshold is set to minimize the variance of these two distributions. 8-Connected components are labeled and counted. This identifies the area covered by each nucleus, and sets each individual nucleus's mask.

Detection and Count of Positive State

The 8-connected components of the nucleus mask image are labeled. The image where Positive State cells are luminescently labeled is background compensated by subtraction of the local average. Positive State cells are then selected by means of a fixed or

adaptive threshold (selection of pixels above Positive State detection threshold). The positive cells are then identified by either the "Morphological" or "Blob Analysis" method:

a. Morphological method (**Figure 34**): A morphological dilation (of 5 pixels for example) is applied to the selected areas. The selected area is logically "AND"ed with the nucleus mask and then the 8-connected components of the resulting area are labeled and counted.

b. Blob Analysis Method (**Figure 35**): The selected area is logically "AND"ed with each separate 8-connected component of the nucleus mask. The area of the resulting image is compared with a threshold (rejection threshold), and if larger, the cell is counted as Positive State.

Linking Positive State Cells (e.g. neurons) to Neurite Outgrowth Assays

For each well the number of detected nuclei and the number of nuclei in the Positive State (i.e. that are neurons) are saved and reported. The total integrated intensity and the average intensity per pixel can also be reported. Next, the neurite outgrowth methods are applied to the Positive State neuronal cells to quantify and characterize their neurite outgrowth. The Positive State nuclei are used to index and track the Positive State cells.

Measuring Degree of Neurite Outgrowth

To measure the degree of neurite outgrowth, both the perimeter and the area of the neuronal cell or cell cluster are measured. To quantify the degree of neurite outgrowth, we use the Form Factor (FF), as discussed above. A summary of the series of steps involved is as follows:

1. Background Compensation: Same as in preferred embodiment.
2. Image Binarization: The image is then binarized to generate a mask image with all the selected cells. The skeleton of the mask is then also computed.
3. Degree of Outgrowth: Connected components in the binarized image are labeled, each of them representing an individual cell or a cell cluster. The perimeter, area, and form factor of each component is computed.
4. Branch Identification: To compute all the branches, first the cell branches are removed by applying a morphological opening (e.g. an image processing erosion of the image) to the original mask image. Then, from the skeletonized mask image (computed above) the region's main cell bodies are subtracted. This leaves only the skeleton of cell branches.

5. Triple-Point Identification: A triple point is the junction where a single branch splits into two or multiple branches, or different neurite branches intersect This image is analyzed to find and count triple points. These points are then removed from the image, thus separating each branch and its sub-branches. A connected components labeling is used to count the number of branches and sub-branches and, by counting the number of pixels of each object (branch), the length of each separate branch is also computed.

Triple Point Characterization: Image acquisition can be expanded to include a feature that further characterizes the triple points. As mentioned above, the triple points may be places where neurites from different cells intersect. If a connection is made between these different neurites, certain proteins that are characteristic of these connections may be expressed. Examples may be synaptic vesicle proteins such as synaptobrevin. Immunofluorescence against these proteins using a fluorophore with a spectra distinguishable from other used in the assay allows determination of whether a connection has been made. Comparison with the neuronal luminescent label to determine whether a triple point is indeed co-localized with the immunofluorescence against the protein characterizes the triple point and measures and quantifies whether inter-neurite connections are being made.

Validation data using the alternative image acquisition embodiment

1. Measurement of Neurite Outgrowth in PC12 cells

PC12 cells were grown on 96-well microplates whose wells had been coated with collagen. Some of the wells contained NGF (nerve growth factor) (0.5-1 μg/ml) to stimulate neurite outgrowth. A control population did not contain NGF. After two days, the cells were labeled with CMFDA according to the manufacturer's instructions. The cells were then fixed in 3.7% formaldehyde for 10 minutes, and the fixative solution also contained 10 μg/ml Hoechst 33342 to label their nuclei. The cells were imaged on the cell screening system of the present invention and then analyzed with a prototype method described above. First, the cells From Factors were calculated.

Condition	Mean form factor +/- sem
+ NGF	18.43 +/1 4.57
-NGF	1.33 +/- 0.05

The results from applying the neurite-outgrowth method to a cluster of cells that had been treated with NGF returned the following analysis for on cell cluster:

Property	Result
Number of cell bodies	3
Degree of neurite outgrowth (form factor)	70.24
# neurites and neurite segments	5
# branch points (i.e.: triple points)	5
Neurite segment length (in μm)	29, 44, 67, 78, and 92

Example 13. Additional Screens

Translocation between the plasma membrane and the cytoplasm:

Profilactin complex dissociation and binding of profilin to the plasma membrane. In one embodiment, a fluorescent protein biosensor of profilin membrane binding is prepared by labeling purified profilin (Federov et al.(1994), *J. Molec. Biol.* 241:480-482; Lanbrechts et al. (1995), *Eur. J. Biochem.* 230:281-286) with a probe possessing a fluorescence lifetime in the range of 2-300 ns. The labeled profilin is introduced into living indicator cells using bulk loading methodology and the indicator cells are treated with test compounds. Fluorescence anisotropy imaging microscopy (Gough and Taylor (1993), *J. Cell Biol.* 121:1095-1107) is used to measure test-compound dependent movement of the fluorescent derivative of profilin between the cytoplasm and membrane for a period of time after treatment ranging from 0.1 s to 10 h.

Rho-RhoGDI complex translocation to the membrane. In another embodiment, indicator cells are treated with test compounds and then fixed, washed, and permeabilized. The indicator cell plasma membrane, cytoplasm, and nucleus are all labeled with distinctly colored markers followed by immunolocalization of Rho protein (Self et al. (1995), *Methods in Enzymology* 256:3-10; Tanaka et al. (1995), *Methods in Enzymology* 256:41-49) with antibodies labeled with a fourth color. Each of the four labels is imaged separately using the cell screening system, and the images used to calculate the amount of inhibition or activation of translocation effected by the test compound. To do this calculation, the images of the probes used to mark the plasma membrane and cytoplasm are used to mask the image of the immunological probe marking the location of intracellular Rho protein. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing

the plasma membrane integrated brightness/area by the cytoplasmic integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound.

β-Arrestin translocation to the plasma membrane upon G-protein receptor activation.

In another embodiment of a cytoplasm to membrane translocation high-content screen, the translocation of β-arrestin protein from the cytoplasm to the plasma membrane is measured in response to cell treatment. To measure the translocation, living indicator cells containing luminescent domain markers are treated with test compounds and the movement of the β-arrestin marker is measured in time and space using the cell screening system of the present invention. In a preferred embodiment, the indicator cells contain luminescent markers consisting of a green fluorescent protein β-arrestin (GFP-β-arrestin) protein chimera (Barak et al. (1997), *J. Biol. Chem.* 272:27497-27500; Daaka et al. (1998), *J. Biol. Chem.* 273:685-688) that is expressed by the indicator cells through the use of transient or stable cell transfection and other reporters used to mark cytoplasmic and membrane domains. When the indicator cells are in the resting state, the domain marker molecules partition predominately in the plasma membrane or in the cytoplasm. In the high-content screen, these markers are used to delineate the cell cytoplasm and plasma membrane in distinct channels of fluorescence. When the indicator cells are treated with a test compound, the dynamic redistribution of the GFP-β-arrestin is recorded as a series of images over a time scale ranging from 0.1 s to 10 h. In a preferred embodiment, the time scale is 1 h. Each image is analyzed by a method that quantifies the movement of the GFP-β-arrestin protein chimera between the plasma membrane and the cytoplasm. To do this calculation, the images of the probes used to mark the plasma membrane and cytoplasm are used to mask the image of the GFP-β-arrestin probe marking the location of intracellular GFP-β-arrestin protein. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the plasma membrane integrated brightness/area by the cytoplasmic integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound. The output of the high-content screen relates quantitative data describing the magnitude of the translocation within a large number of individual cells that have been treated with test compounds of interest.

Translocation between the endoplasmic reticulum and the Golgi:

In one embodiment of an endoplasmic reticulum to Golgi translocation high-content screen, the translocation of a VSVG protein from the ts045 mutant strain of vesicular stomatitis virus (Ellenberg et al. (1997), *J. Cell Biol.* 138:1193-1206; Presley et al. (1997) *Nature* 389:81-85) from the endoplasmic reticulum to the Golgi domain is measured in response to cell treatment. To measure the translocation, indicator cells containing luminescent reporters are treated with test compounds and the movement of the reporters is measured in space and time using the cell screening system of the present invention. The indicator cells contain luminescent reporters consisting of a GFP-VSVG protein chimera that is expressed by the indicator cell through the use of transient or stable cell transfection and other domain markers used to measure the localization of the endoplasmic reticulum and Golgi domains. When the indicator cells are in their resting state at 40°C, the GFP-VSVG protein chimera molecules are partitioned predominately in the endoplasmic reticulum. In this high-content screen, domain markers of distinct colors used to delineate the endoplasmic reticulum and the Golgi domains in distinct channels of fluorescence. When the indicator cells are treated with a test compound and the temperature is simultaneously lowered to 32°C, the dynamic redistribution of the GFP-VSVG protein chimera is recorded as a series of images over a time scale ranging from 0.1 s to 10 h. Each image is analyzed by a method that quantifies the movement of the GFP-VSVG protein chimera between the endoplasmic reticulum and the Golgi domains. To do this calculation, the images of the probes used to mark the endoplasmic reticulum and the Golgi domains are used to mask the image of the GFP-VSVG probe marking the location of intracellular GFP-VSVG protein. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the endoplasmic reticulum integrated brightness/area by the Golgi integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound. The output of the high-content screen relates quantitative data describing the magnitude of the translocation within a large number of individual cells that have been treated with test compounds of interest at final concentrations ranging from 10^{-12} M to 10^{-3} M for a period ranging from 1 min to 10 h.

Induction and inhibition of organellar function:

Intracellular microtubule stability. In one embodiment of an organellar function high-content screen, the assembly state of intracellular microtubules is measured in response to cell treatment. To measure microtubule assembly state, indicator cells containing

luminescent reporters are treated with test compounds and the distribution of the reporters is measured in space and time using the cell screening system of the present invention.

In a preferred embodiment, the reporter of intracellular microtubule assembly is MAP 4 (Bulinski et al. (1997), *J. Cell Science* 110:3055-3064), a ubiquitous microtubule associated protein that is known to interact with microtubules in interphase and mitotic cells. The indicator cells contain luminescent reporters consisting of a GFP-MAP 4 chimera that is expressed by the indicator cells through the use of transient or stable cell transfection and other reporters are used to measure the localization of the cytoplasmic and membrane components. A GFP-MAP 4 construct is prepared as follows: PCR amplification of native or mutant GFP molecules using primers to introduce restriction enzyme sites is performed. The PCR product is ligated into the MAP 4 cDNA within a eukaryotic expression vector. Indicator cells are then transfected with the expression vector to produce either transiently or stably transfected indicator cells.

Indicator cells are treated with test compounds at final concentrations ranging from 10^{-12} M to 10^{-3} M for a period ranging from 1 min to 10 h. Growth medium containing labeling reagent to mark the nucleus and the cytoplasm are added. After incubation, the cells are washed with Hank's balanced salt solution (HBSS), fixed with 3.7% formaldehyde for 10 min at room temperature, and washed and stored in HBSS.

Image data are obtained from both fixed and living indicator cells. To extract morphometric data from each of the images obtained the following method of analysis is used:

1. Threshold each nucleus and cytoplasmic image to produce a mask that has value = 0 for each pixel outside a nucleus or cell boundary.
2. Overlay the mask on the original image, detect each object in the field (*i.e.*, nucleus or cell), and calculate its size, shape, and integrated intensity.
3. Overlay the whole cell mask obtained above on the corresponding GFP-MAP 4 image and use an automated measurement of edge strength routine (Kolega et al. (1993). *BioImaging* 1:136-150) to calculate the total edge strength within each cell. To normalize for cell size, the total edge strength is divided by the cell area to give a "fibrousness" value. Large fibrousness values are associated with strong edge strength values and are therefore maximal in cells containing distinct microtubule structures. Likewise, small fibrousness values are associated with weak edge strength and are minimal in cells with depolymerized microtubules. The physiological range of fibrousness values is set by

treating cells with either the microtubule stabilizing drug paclitaxel (10 μM) or the microtubule depolymerizing drug nocodazole (10 μg/ml).

High-content screens involving the functional localization of macromolecules

Within this class of high-content screen, the functional localization of macromolecules in response to external stimuli is measured within living cells.

Glycolytic enzyme activity regulation. In a preferred embodiment of a cellular enzyme activity high-content screen, the activity of key glycolytic regulatory enzymes are measured in treated cells. To measure enzyme activity, indicator cells containing luminescent labeling reagents are treated with test compounds and the activity of the reporters is measured in space and time using cell screening system of the present invention.

In one embodiment, the reporter of intracellular enzyme activity is fructose-6-phosphate, 2-kinase/fructose-2,6-bisphosphatase (PFK-2), a regulatory enzyme whose phosphorylation state indicates intracellular carbohydrate anabolism or catabolism (Deprez et al. (1997) *J. Biol. Chem.* 272:17269-17275; Kealer et al. (1996) *FEBS Letters* 395:225-227; Lee et al. (1996), *Biochemistry* 35:6010-6019). The indicator cells contain luminescent reporters consisting of a fluorescent protein biosensor of PFK-2 phosphorylation. The fluorescent protein biosensor is constructed by introducing an environmentally sensitive fluorescent dye near to the known phosphorylation site of the enzyme (Deprez et al. (1997), *supra*; Giuliano et al. (1995), *supra*). The dye can be of the ketocyanine class (Kessler and Wolfbeis (1991), *Spectrochimica Acta* 47A:187-192) or any class that contains a protein reactive moiety and a fluorochrome whose excitation or emission spectrum is sensitive to solution polarity. The fluorescent protein biosensor is introduced into the indicator cells using bulk loading methodology.

Living indicator cells are treated with test compounds, at final concentrations ranging from 10^{-12} M to 10^{-3} M for times ranging from 0.1 s to 10 h. In a preferred embodiment, ratio image data are obtained from living treated indicator cells by collecting a spectral pair of fluorescence images at each time point. To extract morphometric data from each time point, a ratio is made between each pair of images by numerically dividing the two spectral images at each time point, pixel by pixel. Each pixel value is then used to calculate the fractional phosphorylation of PFK-2. At small fractional values of phosphorylation, PFK-2 stimulates carbohydrate catabolism. At high fractional values of phosphorylation, PFK-2 stimulates carbohydrate anabolism.

Protein kinase A activity and localization of subunits. In another embodiment of a high-content screen, both the domain localization and activity of protein kinase A (PKA) within indicator cells are measured in response to treatment with test compounds.

The indicator cells contain luminescent reporters including a fluorescent protein biosensor of PKA activation. The fluorescent protein biosensor is constructed by introducing an environmentally sensitive fluorescent dye into the catalytic subunit of PKA near the site known to interact with the regulatory subunit of PKA (Harootunian et al. (1993), *Mol. Biol. of the Cell* 4:993-1002; Johnson et al. (1996), *Cell* 85:149-158; Giuliano et al. (1995), *supra*). The dye can be of the ketocyanine class (Kessler, and Wolfbeis (1991), *Spectrochimica Acta* 47A:187-192) or any class that contains a protein reactive moiety and a fluorochrome whose excitation or emission spectrum is sensitive to solution polarity. The fluorescent protein biosensor of PKA activation is introduced into the indicator cells using bulk loading methodology.

In one embodiment, living indicator cells are treated with test compounds, at final concentrations ranging from 10^{-12} M to 10^{-3} M for times ranging from 0.1 s to 10 h. In a preferred embodiment, ratio image data are obtained from living treated indicator cells. To extract biosensor data from each time point, a ratio is made between each pair of images, and each pixel value is then used to calculate the fractional activation of PKA (*e.g.*, separation of the catalytic and regulatory subunits after cAMP binding). At high fractional values of activity, PFK-2 stimulates biochemical cascades within the living cell.

To measure the translocation of the catalytic subunit of PKA, indicator cells containing luminescent reporters are treated with test compounds and the movement of the reporters is measured in space and time using the cell screening system. The indicator cells contain luminescent reporters consisting of domain markers used to measure the localization of the cytoplasmic and nuclear domains. When the indicator cells are treated with a test compounds, the dynamic redistribution of a PKA fluorescent protein biosensor is recorded intracellularly as a series of images over a time scale ranging from 0.1 s to 10 h. Each image is analyzed by a method that quantifies the movement of the PKA between the cytoplasmic and nuclear domains. To do this calculation, the images of the probes used to mark the cytoplasmic and nuclear domains are used to mask the image of the PKA fluorescent protein biosensor. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the cytoplasmic integrated brightness/area by the nuclear

integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound. The output of the high-content screen relates quantitative data describing the magnitude of the translocation within a large number of individual cells that have been treated with test compound in the concentration range of 10^{-12} M to 10^{-3} M.

High-content screens involving the induction or inhibition of gene expression

RNA-based fluorescent biosensors

Cytoskeletal protein transcription and message localization. Regulation of the general classes of cell physiological responses including cell-substrate adhesion, cell-cell adhesion, signal transduction, cell-cycle events, intermediary and signaling molecule metabolism, cell locomotion, cell-cell communication, and cell death can involve the alteration of gene expression. High-content screens can also be designed to measure this class of physiological response.

In one embodiment, the reporter of intracellular gene expression is an oligonucleotide that can hybridize with the target mRNA and alter its fluorescence signal. In a preferred embodiment, the oligonucleotide is a molecular beacon (Tyagi and Kramer (1996) *Nat. Biotechnol.* 14:303-308), a luminescence-based reagent whose fluorescence signal is dependent on intermolecular and intramolecular interactions. The fluorescent biosensor is constructed by introducing a fluorescence energy transfer pair of fluorescent dyes such that there is one at each end (5' and 3') of the reagent. The dyes can be of any class that contains a protein reactive moiety and fluorochromes whose excitation and emission spectra overlap sufficiently to provide fluorescence energy transfer between the dyes in the resting state, including, but not limited to, fluorescein and rhodamine (Molecular Probes, Inc.). In a preferred embodiment, a portion of the message coding for β-actin (Kislauskis et al. (1994), *J. Cell Biol.* 127:441-451; McCann et al. (1997), *Proc. Natl. Acad. Sci.* 94:5679-5684; Sutoh (1982), *Biochemistry* 21:3654-3661) is inserted into the loop region of a hairpin-shaped oligonucleotide with the ends tethered together due to intramolecular hybridization. At each end of the biosensor a fluorescence donor (fluorescein) and a fluorescence acceptor (rhodamine) are covalently bound. In the tethered state, the fluorescence energy transfer is maximal and therefore indicative of an unhybridized molecule. When hybridized with the mRNA coding for β-actin, the tether is broken and energy transfer is lost. The complete fluorescent biosensor is introduced into the indicator cells using bulk loading methodology.

In one embodiment, living indicator cells are treated with test compounds, at final concentrations ranging from 10^{-12} M to 10^{-3} M for times ranging from 0.1 s to 10 h. In a preferred embodiment, ratio image data are obtained from living treated indicator cells. To extract morphometric data from each time point, a ratio is made between each pair of images, and each pixel value is then used to calculate the fractional hybridization of the labeled nucleotide. At small fractional values of hybridization little expression of β-actin is indicated. At high fractional values of hybridization, maximal expression of β-actin is indicated. Furthermore, the distribution of hybridized molecules within the cytoplasm of the indicator cells is also a measure of the physiological response of the indicator cells.

Cell surface binding of a ligand

Labeled insulin binding to its cell surface receptor in living cells. Cells whose plasma membrane domain has been labeled with a labeling reagent of a particular color are incubated with a solution containing insulin molecules (Lee et al. (1997), *Biochemistry* 36:2701-2708; Martinez-Zaguilan et al. (1996), *Am. J. Physiol.* 270:C1438-C1446) that are labeled with a luminescent probe of a different color for an appropriate time under the appropriate conditions. After incubation, unbound insulin molecules are washed away, the cells fixed and the distribution and concentration of the insulin on the plasma membrane is measured. To do this, the cell membrane image is used as a mask for the insulin image. The integrated intensity from the masked insulin image is compared to a set of images containing known amounts of labeled insulin. The amount of insulin bound to the cell is determined from the standards and used in conjunction with the total concentration of insulin incubated with the cell to calculate a dissociation constant or insulin to its cell surface receptor.

Labeling of cellular compartments

Whole cell labeling

Whole cell labeling is accomplished by labeling cellular components such that dynamics of cell shape and motility of the cell can be measured over time by analyzing fluorescence images of cells.

In one embodiment, small reactive fluorescent molecules are introduced into living cells. These membrane-permeant molecules both diffuse through and react with protein components in the plasma membrane. Dye molecules react with intracellular molecules to

both increase the fluorescence signal emitted from each molecule and to entrap the fluorescent dye within living cells. These molecules include reactive chloromethyl derivatives of aminocoumarins, hydroxycoumarins, eosin diacetate, fluorescein diacetate, some Bodipy dye derivatives, and tetramethylrhodamine. The reactivity of these dyes toward macromolecules includes free primary amino groups and free sulfhydryl groups.

In another embodiment, the cell surface is labeled by allowing the cell to interact with fluorescently labeled antibodies or lectins (Sigma Chemical Company, St. Louis, MO) that react specifically with molecules on the cell surface. Cell surface protein chimeras expressed by the cell of interest that contain a green fluorescent protein, or mutant thereof, component can also be used to fluorescently label the entire cell surface. Once the entire cell is labeled, images of the entire cell or cell array can become a parameter in high content screens, involving the measurement of cell shape, motility, size, and growth and division.

Plasma membrane labeling

In one embodiment, labeling the whole plasma membrane employs some of the same methodology described above for labeling the entire cells. Luminescent molecules that label the entire cell surface act to delineate the plasma membrane.

In a second embodiment subdomains of the plasma membrane, the extracellular surface, the lipid bilayer, and the intracellular surface can be labeled separately and used as components of high content screens. In the first embodiment, the extracellular surface is labeled using a brief treatment with a reactive fluorescent molecule such as the succinimidyl ester or iodoacetamde derivatives of fluorescent dyes such as the fluoresceins, rhodamines, cyanines, and Bodipys.

In a third embodiment, the extracellular surface is labeled using fluorescently labeled macromolecules with a high affinity for cell surface molecules. These include fluorescently labeled lectins such as the fluorescein, rhodamine, and cyanine derivatives of lectins derived from jack bean (Con A), red kidney bean (erythroagglutinin PHA-E), or wheat germ.

In a fourth embodiment, fluorescently labeled antibodies with a high affinity for cell surface components are used to label the extracellular region of the plasma membrane. Extracellular regions of cell surface receptors and ion channels are examples of proteins that can be labeled with antibodies.

In a fifth embodiment, the lipid bilayer of the plasma membrane is labeled with fluorescent molecules. These molecules include fluorescent dyes attached to long chain

hydrophobic molecules that interact strongly with the hydrophobic region in the center of the plasma membrane lipid bilayer. Examples of these dyes include the PKH series of dyes (U.S. 4,783,401, 4,762701, and 4,859,584; available commercially from Sigma Chemical Company, St. Louis, MO), fluorescent phospholipids such as nitrobenzoxadiazole glycerophosphoethanolamine and fluorescein-derivatized dihexadecanoylglycerophosphoetha-nolamine, fluorescent fatty acids such as 5-butyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-nonanoic acid and 1-pyrenedecanoic acid (Molecular Probes, Inc.), fluorescent sterols including cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate and cholesteryl 1-pyrenehexanoate, and fluorescently labeled proteins that interact specifically with lipid bilayer components such as the fluorescein derivative of annexin V (Caltag Antibody Co, Burlingame, CA).

In another embodiment, the intracellular component of the plasma membrane is labeled with fluorescent molecules. Examples of these molecules are the intracellular components of the trimeric G-protein receptor, adenylyl cyclase, and ionic transport proteins. These molecules can be labeled as a result of tight binding to a fluorescently labeled specific antibody or by the incorporation of a fluorescent protein chimera that is comprised of a membrane-associated protein and the green fluorescent protein, and mutants thereof.

Endosome fluorescence labeling

In one embodiment, ligands that are transported into cells by receptor-mediated endocytosis are used to trace the dynamics of endosomal organelles. Examples of labeled ligands include Bodipy FL-labeled low density lipoprotein complexes, tetramethylrhodamine transferrin analogs, and fluorescently labeled epidermal growth factor (Molecular Probes, Inc.)

In a second embodiment, fluorescently labeled primary or secondary antibodies (Sigma Chemical Co. St. Louis, MO; Molecular Probes, Inc. Eugene, OR; Caltag Antibody Co.) that specifically label endosomal ligands are used to mark the endosomal compartment in cells.

In a third embodiment, endosomes are fluorescently labeled in cells expressing protein chimeras formed by fusing a green fluorescent protein, or mutants thereof, with a receptor whose internalization labels endosomes. Chimeras of the EGF, transferrin, and low density lipoprotein receptors are examples of these molecules.

Lysosome labeling

In one embodiment, membrane permeant lysosome-specific luminescent reagents are used to label the lysosomal compartment of living and fixed cells. These reagents include the luminescent molecules neutral red, N-(3-((2,4-dinitrophenyl)amino)propyl)-N-(3-aminopropyl)methylamine, and the LysoTracker probes which report intralysosomal pH as well as the dynamic distribution of lysosomes (Molecular Probes, Inc.)

In a second embodiment, antibodies against lysosomal antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label lysosomal components that are localized in specific lysosomal domains. Examples of these components are the degradative enzymes involved in cholesterol ester hydrolysis, membrane protein proteases, and nucleases as well as the ATP-driven lysosomal proton pump.

In a third embodiment, protein chimeras consisting of a lysosomal protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the lysosomal domain. Examples of these components are the degradative enzymes involved in cholesterol ester hydrolysis, membrane protein proteases, and nucleases as well as the ATP-driven lysosomal proton pump.

Cytoplasmic fluorescence labeling

In one embodiment, cell permeant fluorescent dyes (Molecular Probes, Inc.) with a reactive group are reacted with living cells. Reactive dyes including monobromobimane, 5-chloromethylfluorescein diacetate, carboxy fluorescein diacetate succinimidyl ester, and chloromethyl tetramethylrhodamine are examples of cell permeant fluorescent dyes that are used for long term labeling of the cytoplasm of cells.

In a second embodiment, polar tracer molecules such as Lucifer yellow and cascade blue-based fluorescent dyes (Molecular Probes, Inc.) are introduced into cells using bulk loading methods and are also used for cytoplasmic labeling.

In a third embodiment, antibodies against cytoplasmic components (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to fluorescently label the cytoplasm. Examples of cytoplasmic antigens are many of the enzymes involved in intermediary metabolism. Enolase, phosphofructokinase, and acetyl-CoA dehydrogenase are examples of uniformly distributed cytoplasmic antigens.

In a fourth embodiment, protein chimeras consisting of a cytoplasmic protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the cytoplasm. Fluorescent chimeras of uniformly distributed proteins are used to label the entire cytoplasmic domain. Examples of these proteins are many of the proteins involved in intermediary metabolism and include enolase, lactate dehydrogenase, and hexokinase.

In a fifth embodiment, antibodies against cytoplasmic antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label cytoplasmic components that are localized in specific cytoplasmic sub-domains. Examples of these components are the cytoskeletal proteins actin, tubulin, and cytokeratin. A population of these proteins within cells is assembled into discrete structures, which in this case, are fibrous. Fluorescence labeling of these proteins with antibody-based reagents therefore labels a specific sub-domain of the cytoplasm.

In a sixth embodiment, non-antibody-based fluorescently labeled molecules that interact strongly with cytoplasmic proteins are used to label specific cytoplasmic components. One example is a fluorescent analog of the enzyme DNAse I (Molecular Probes, Inc.) Fluorescent analogs of this enzyme bind tightly and specifically to cytoplasmic actin, thus labeling a sub-domain of the cytoplasm. In another example, fluorescent analogs of the mushroom toxin phalloidin or the drug paclitaxel (Molecular Probes, Inc.) are used to label components of the actin- and microtubule-cytoskeletons, respectively.

In a seventh embodiment, protein chimeras consisting of a cytoplasmic protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label specific domains of the cytoplasm. Fluorescent chimeras of highly localized proteins are used to label cytoplasmic sub-domains. Examples of these proteins are many of the proteins involved in regulating the cytoskeleton. They include the structural proteins actin, tubulin, and cytokeratin as well as the regulatory proteins microtubule associated protein 4 and α-actinin.

Nuclear labeling

In one embodiment, membrane permeant nucleic-acid-specific luminescent reagents (Molecular Probes, Inc.) are used to label the nucleus of living and fixed cells. These reagents include cyanine-based dyes (*e.g.*, TOTO®, YOYO®, and BOBO™), phenanthidines and acridines (*e.g.*, ethidium bromide, propidium iodide, and acridine orange), indoles and

imidazoles (*e.g.*, Hoechst 33258, Hoechst 33342, and 4',6-diamidino-2-phenylindole), and other similar reagents (*e.g.*, 7-aminoactinomycin D, hydroxystilbamidine, and the psoralens).

In a second embodiment, antibodies against nuclear antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label nuclear components that are localized in specific nuclear domains. Examples of these components are the macromolecules involved in maintaining DNA structure and function. DNA, RNA, histones, DNA polymerase, RNA polymerase, lamins, and nuclear variants of cytoplasmic proteins such as actin are examples of nuclear antigens.

In a third embodiment, protein chimeras consisting of a nuclear protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the nuclear domain. Examples of these proteins are many of the proteins involved in maintaining DNA structure and function. Histones, DNA polymerase, RNA polymerase, lamins, and nuclear variants of cytoplasmic proteins such as actin are examples of nuclear proteins.

Mitochondrial labeling

In one embodiment, membrane permeant mitochondrial-specific luminescent reagents (Molecular Probes, Inc.) are used to label the mitochondria of living and fixed cells. These reagents include rhodamine 123, tetramethyl rosamine, JC-1, and the MitoTracker reactive dyes.

In a second embodiment, antibodies against mitochondrial antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label mitochondrial components that are localized in specific mitochondrial domains. Examples of these components are the macromolecules involved in maintaining mitochondrial DNA structure and function. DNA, RNA, histones, DNA polymerase, RNA polymerase, and mitochondrial variants of cytoplasmic macromolecules such as mitochondrial tRNA and rRNA are examples mitochondrial antigens. Other examples of mitochondrial antigens are the components of the oxidative phosphorylation system found in the mitochondria (*e.g.*, cytochrome c, cytochrome c oxidase, and succinate dehydrogenase).

In a third embodiment, protein chimeras consisting of a mitochondrial protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the mitochondrial domain. Examples of these components are the macromolecules involved in maintaining mitochondrial DNA structure

and function. Examples include histones, DNA polymerase, RNA polymerase, and the components of the oxidative phosphorylation system found in the mitochondria (*e.g.*, cytochrome c, cytochrome c oxidase, and succinate dehydrogenase).

Endoplasmic reticulum labeling

In one embodiment, membrane permeant endoplasmic reticulum-specific luminescent reagents (Molecular Probes, Inc.) are used to label the endoplasmic reticulum of living and fixed cells. These reagents include short chain carbocyanine dyes (*e.g.*, $DiOC_6$ and $DiOC_3$), long chain carbocyanine dyes (*e.g.*, $DiIC_{16}$ and $DiIC_{18}$), and luminescently labeled lectins such as concanavalin A.

In a second embodiment, antibodies against endoplasmic reticulum antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label endoplasmic reticulum components that are localized in specific endoplasmic reticulum domains. Examples of these components are the macromolecules involved in the fatty acid elongation systems, glucose-6-phosphatase, and HMG CoA-reductase.

In a third embodiment, protein chimeras consisting of a endoplasmic reticulum protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the endoplasmic reticulum domain. Examples of these components are the macromolecules involved in the fatty acid elongation systems, glucose-6-phosphatase, and HMG CoA-reductase.

Golgi labeling

In one embodiment, membrane permeant Golgi-specific luminescent reagents (Molecular Probes, Inc.) are used to label the Golgi of living and fixed cells. These reagents include luminescently labeled macromolecules such as wheat germ agglutinin and Brefeldin A as well as luminescently labeled ceramide.

In a second embodiment, antibodies against Golgi antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label Golgi components that are localized in specific Golgi domains. Examples of these components are N-acetylglucosamine phosphotransferase, Golgi-specific phosphodiesterase, and mannose-6-phosphate receptor protein.

In a third embodiment, protein chimeras consisting of a Golgi protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the Golgi domain. Examples of these components are N-

acetylglucosamine phosphotransferase, Golgi-specific phosphodiesterase, and mannose-6-phosphate receptor protein.

While many of the examples presented involve the measurement of single cellular processes, this is again is intended for purposes of illustration only. Multiple parameter high-content screens can be produced by combining several single parameter screens into a multiparameter high-content screen or by adding cellular parameters to any existing high-content screen. Furthermore, while each example is described as being based on either live or fixed cells, each high-content screen can be designed to be used with both live and fixed cells.

Those skilled in the art will recognize a wide variety of distinct screens that can be developed based on the disclosure provided herein. There is a large and growing list of known biochemical and molecular processes in cells that involve translocations or reorganizations of specific components within cells. The signaling pathway from the cell surface to target sites within the cell involves the translocation of plasma membrane-associated proteins to the cytoplasm. For example, it is known that one of the src family of protein tyrosine kinases, pp60c-src (Walker et al (1993), *J. Biol. Chem.* 268:19552-19558) translocates from the plasma membrane to the cytoplasm upon stimulation of fibroblasts with platelet-derived growth factor (PDGF). Additionally, the targets for screening can themselves be converted into fluorescence-based reagents that report molecular changes including ligand-binding and post-translocational modifications.

We claim:

1. An automated method for measuring cell viability, comprising

-providing an array of locations comprising multiple cells;

-contacting the cells with a luminescently labeled cell identification reporter molecule that labels all cells;

-contacting the cells with a first luminescently labeled cell state reporter molecule that is specific for either

(a) live cells, or

(b) dead cells;

wherein emission from the luminescently labeled cell identification and first cell state reporter molecules are distinguishable;

-imaging multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the luminescently-labeled cell identification and first reporter molecules;

-converting the luminescent signals into digital data; and

-utilizing the digital data to automatically make measurements, wherein the measurements are used to automatically calculate a percentage of cells that are viable.

2. The method of claim 1 wherein the luminescent cell identification reporter molecule comprises a membrane permeable nucleic acid stain.

3. The method of claim 1 wherein the first luminescent cell state reporter molecule comprises a plasma membrane permeable marker of live cell activity.

4. The method of claim 1 wherein the first luminescent cell state reporter molecule is specific for dead cells and comprises a membrane-impermeant nucleic acid stain.

5. The method of claim 1, wherein the imaging comprises:

(a) producing a nuclear mask; and

(b) producing a positive state mask.

6. The method of claim 5 wherein the imaging further comprises measuring the size of the overlap area between the nuclear mask and the positive state mask.

7. The method of claim 5 wherein the imaging further comprises performing morphological operations on at least one of

(a) the nuclear mask; or

(b) the positive state mask.

8. The method of claim 1 wherein the measurements comprise determining

(a) a number of cells labeled with the luminescent cell identification reporter molecule; and at least one of the following:

(b) a number of cells labeled with the first luminescent cell state reporter molecule; (c) a percentage of cells labeled with the first luminescent cell state reporter molecule;

(d) a normalized intensity of luminescent signals from the luminescent cell identification reporter molecule in a nuclear mask;

(e) total nuclear area; or

(f) average nuclear area.

9. The method of claim 1, further comprising contacting the cells with a second luminescently labeled cell state reporter molecule that is specific for either

(a) dead cells if the first luminescently labeled cell state reporter molecule is specific for live cells; or

(b) live cells if the first luminescently labeled cell state reporter molecule is specific for dead cells.

10. The method of claim 9, wherein the imaging comprises:

(a) producing a nuclear mask; and

(b) producing a first positive state mask for the first luminescent cell state reporter molecule; and

(c) producing a second positive state mask for the second luminescent cell state reporter molecule.

11. The method of claim 10 wherein the imaging further comprises measuring the size of the overlap area between the nuclear mask and at least one of

(a) the first positive state mask; or

(b) the second positive state mask.

12. The method of claim 10 wherein the imaging further comprises performing morphological operations on one or more of

(a) the nuclear mask;

(b) the first positive state mask; or

(c) the second positive state mask.

13. The method of claim 9 where the measurements comprise determining

(a) a number of cells labeled with the luminescent cell identification reporter molecule;

(b) at least one of the following:

(i) a number of cells labeled with the first luminescent cell state reporter molecule,

(ii) a percentage of cells labeled with the first luminescent cell state reporter molecule, or

(iii) a normalized intensity of luminescent signals from the first luminescent cell state reporter molecule in a nuclear mask; and

(c) at least one of the following:

(i) a number of cells labeled with the second luminescent cell state reporter molecule,

(ii) a percentage of cells labeled with the second luminescent cell state reporter molecule, or

(iii) a normalized intensity of luminescent signals from the second luminescent cell state reporter molecule in a nuclear mask

14. The method of claim 13 wherein the measurements further comprise at least one of the following:

(a) a number of cells labeled with the first luminescent cell state reporter molecule that are not labeled with the second luminescent reporter molecule,

(b) a percentage of cells labeled with the first luminescent cell state reporter molecule that are not labeled with the second luminescent reporter molecule,

(c) a number of cells labeled with the second luminescent cell state reporter molecule that are not labeled with the first luminescent reporter molecule,

(d) a percentage of cells labeled with the second luminescent cell state reporter molecule that are not labeled with the first luminescent reporter molecule,

(e) a number of cells labeled with both the first luminescent cell state reporter molecule and the second luminescent reporter molecule, or

(f) a percentage of cells labeled with both the first luminescent cell state reporter molecule and the second luminescent reporter molecule.

15. The method of claim 1 further comprising contacting the cells with a test compound, wherein the measurements are used to automatically calculate an effect of the test compound on cell viability.

16. The method of claim 15 wherein the effect is selected from the group consisting of an LD50 dosage of the test compound, a dose-response of the cells to the compound, or a time course of the effect of the compound on the cells.

17. The method of claim 1 further comprising fixing the cells prior to imaging.

18. The method of claim 1, wherein sub-regions of the array of locations containing cells are sampled multiple times at intervals to provide kinetic measurement changes in cell viability

19. A computer readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute the method of claim 1, wherein the cell screening system comprises an optical system with a stage adapted for holding a plate containing cells, a digital camera, a device for directing light emitted from the cells to the digital camera, and a computer for receiving and processing the digital data from the digital camera.

20. A method for the characterization of a cell state of individual cells within a cell population, comprising

(a) providing an array of locations containing cells, wherein the cells possess a luminescently labeled cell identification reporter molecule and one or more luminescently labeled cell state reporter molecules, wherein emission from the cell identification and the cell state luminescently labeled reporter molecules are distinguishable;

(b) imaging multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the cell identification and the cell state reporter molecules;

(c) converting the luminescent signals into digital data;

(d) using the digital data to create a mask of the cell identification reporter molecule and the cell state reporter molecule; and

(e) determining the intensity of the cell state reporter molecule mask that co-localizes with the cell identification reporter molecule mask, wherein if this intensity is above or below a certain threshold, the cell is in a particular physiological state.

21. The method of claim 20 wherein the imaging further comprises performing morphological operations on at least one of

(a) the cell identification mask; or

(b) the cell state mask.

22. The method of claim 20 wherein the measurements comprise determining

(a) a number of cells labeled with the luminescent cell identification reporter molecule; and at least one of the following:

(b) a number of cells labeled with the luminescent cell state reporter molecule;

(c) a percentage of cells labeled with the luminescent cell state reporter molecule;

(d) a normalized intensity of luminescent signals from the luminescent cell identification reporter molecule in a nuclear mask;

(e) total nuclear area; or

(f) average nuclear area.

23. The method of claim 22, wherein the physiological state detected is selected from the group consisting of cell viability, pathogenic infection of cells, receptor internalization, cell type, adipogenesis, and foam cell formation.

Figure 1

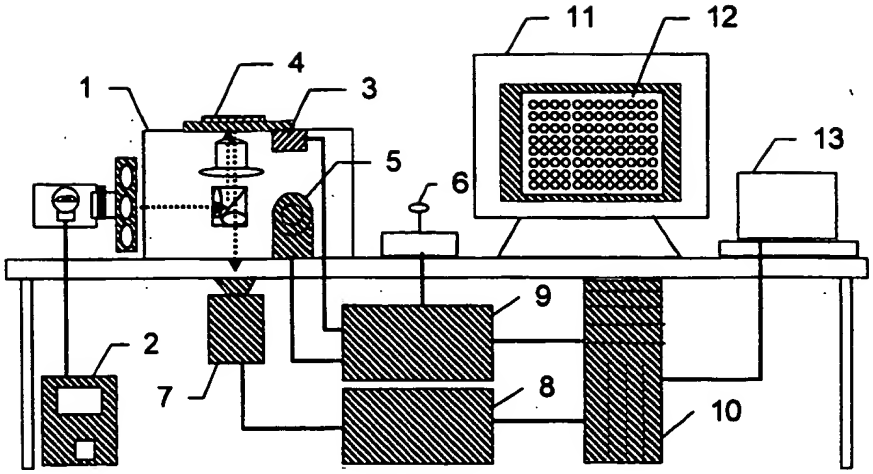
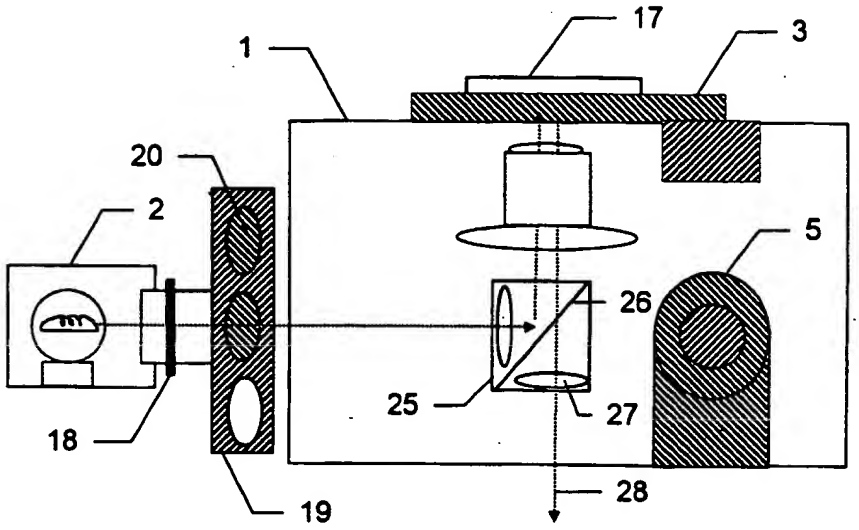


Figure 2



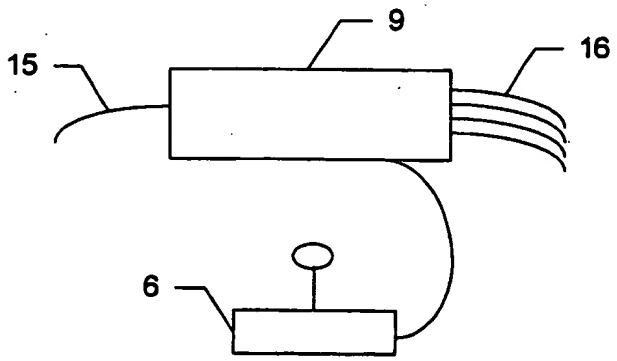


Figure 3

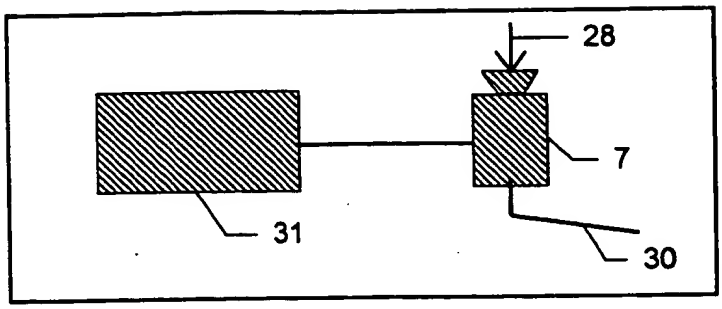


Figure 4

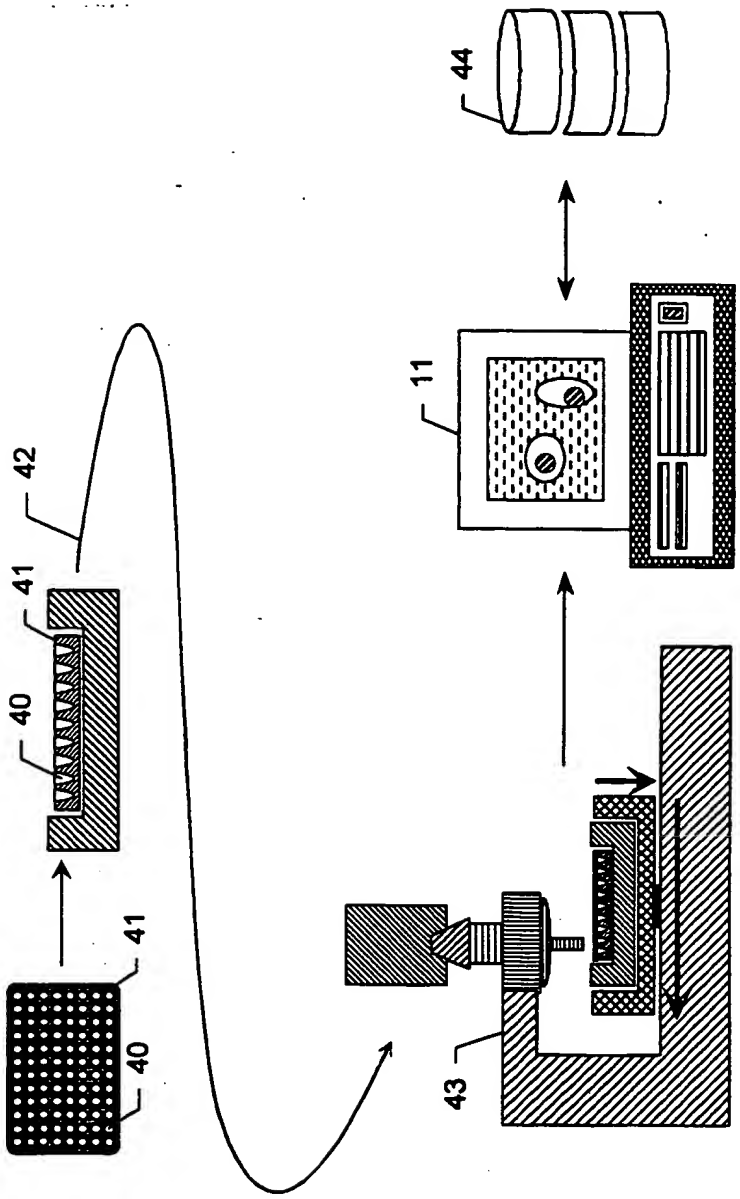
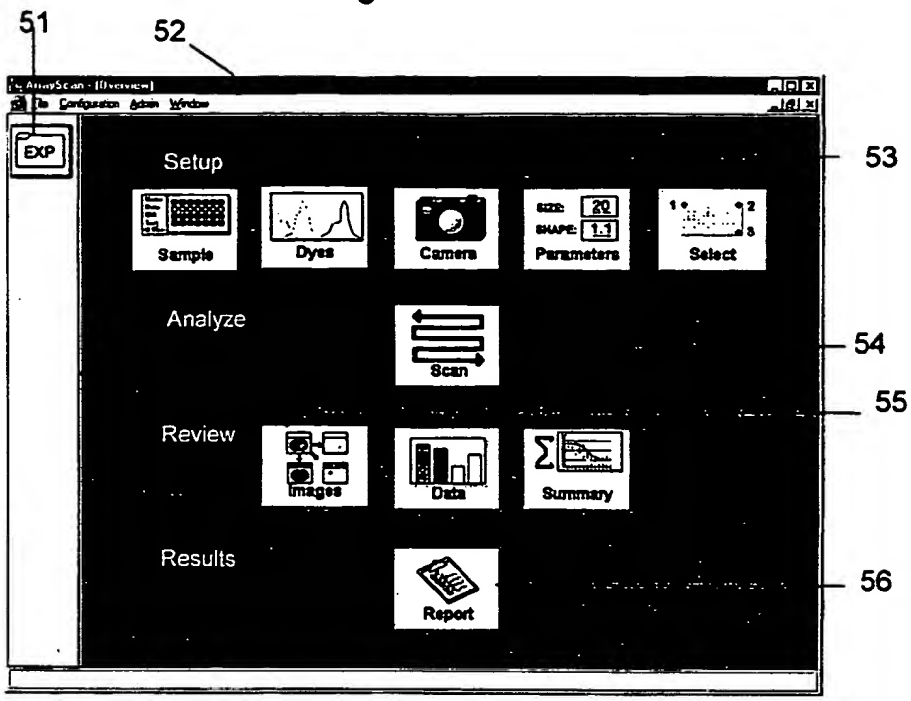


Figure 5


51
52
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Sample
Dyes
Camera
SIZE: 20
SHAPE: 1.1
Parameters
Select
53
Analyze
Scan
54
55
Review
Images
Data
Summary
Results
Report
56

Figure 6

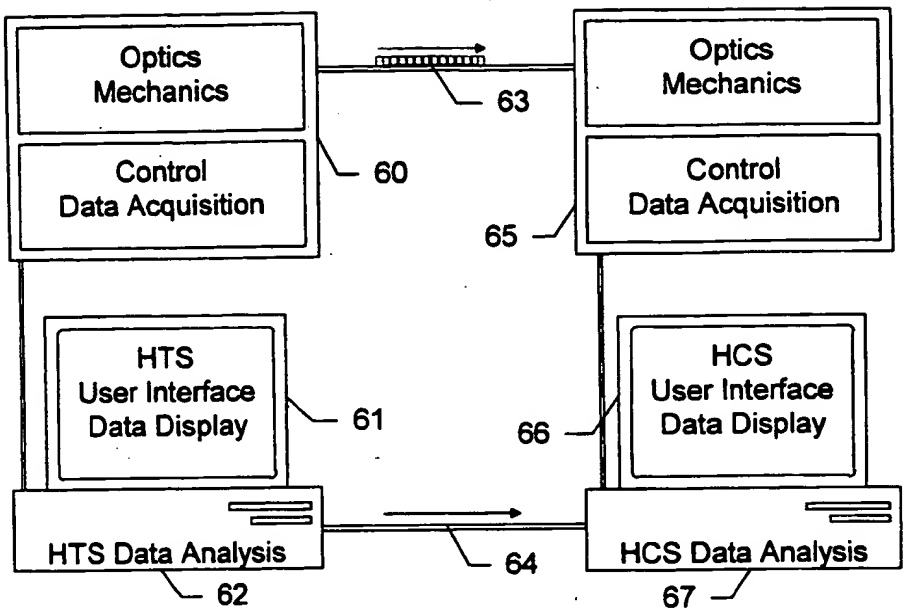

Optics
Mechanics
Control
Data Acquisition
60
63
Optics
Mechanics
Control
Data Acquisition
65
HTS
User Interface
Data Display
61
66
HCS
User Interface
Data Display
HTS Data Analysis
62
64
HCS Data Analysis
67

Figure 7

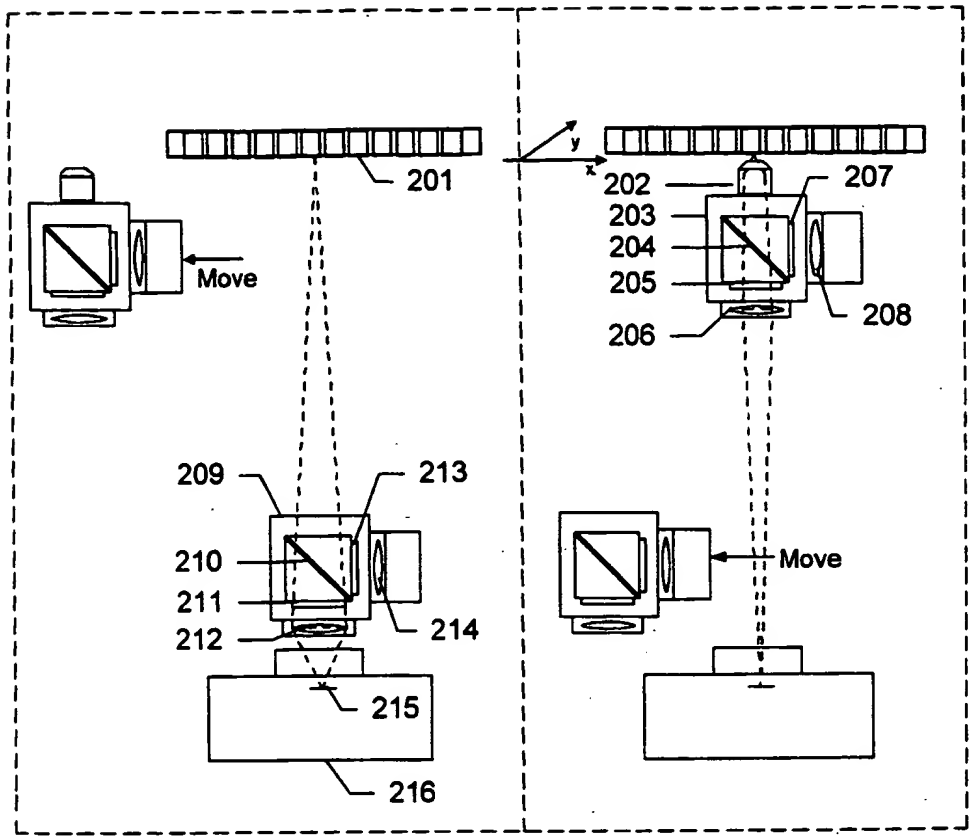

201
Move
209
213
210
211
212
214
215
216
y
x
202
207
203
204
205
208
206
Move

Figure 8

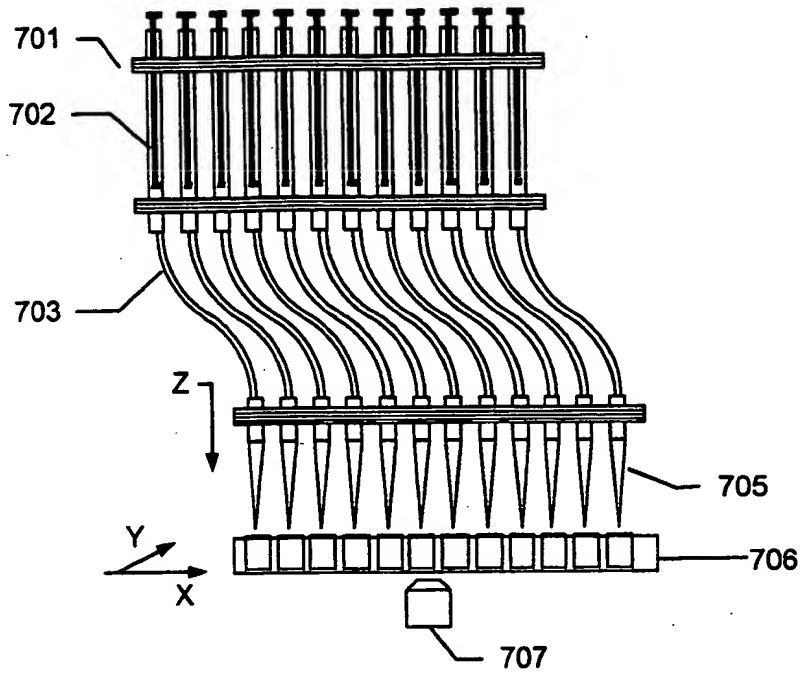


Figure 9

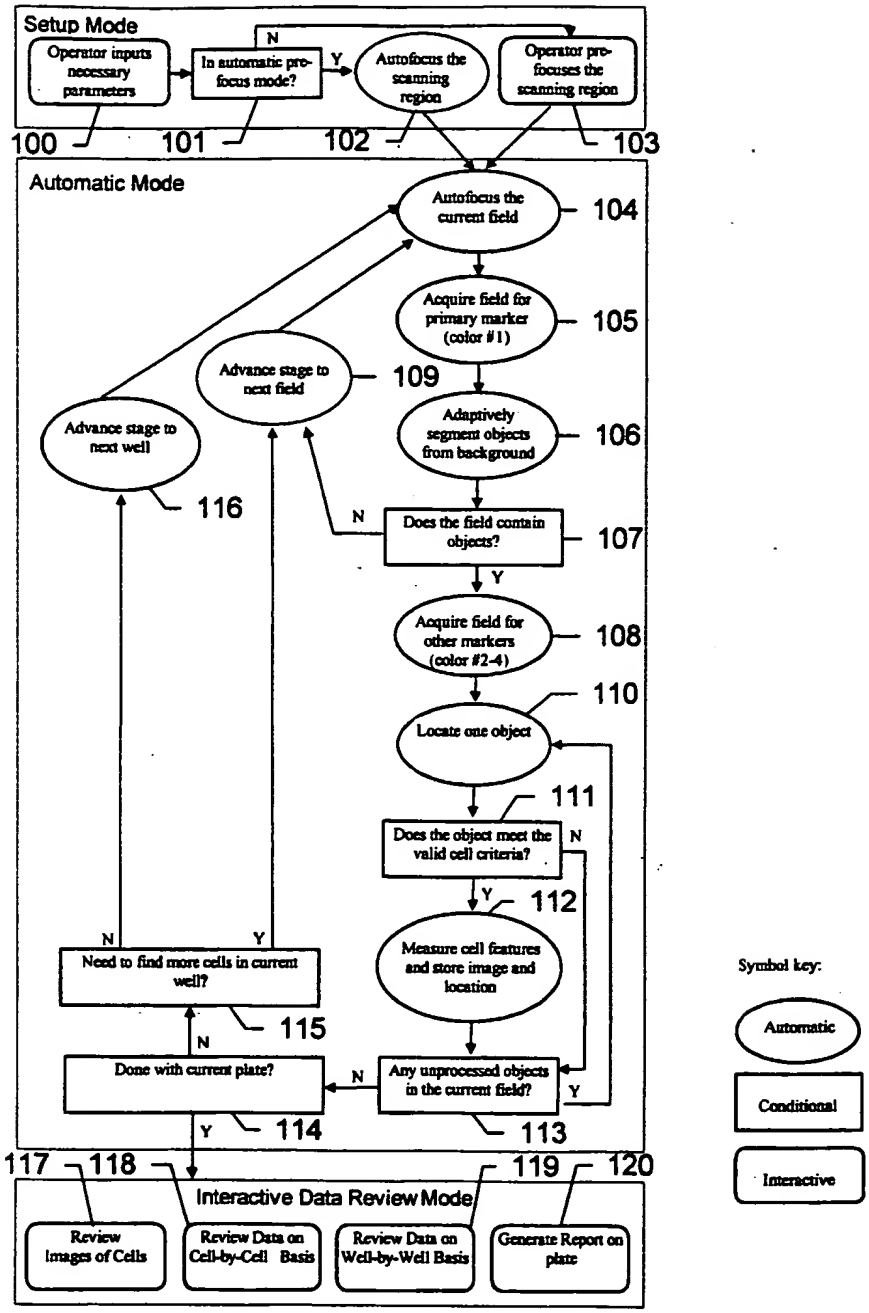

Setup Mode
Operator inputs necessary parameters
In automatic pre-focus mode?
N
Y
Autofocus the scanning region
Operator pre-focuses the scanning region
100
101
102
103
Automatic Mode
Autofocus the current field
104
Acquire field for primary marker (color #1)
105
Advance stage to next field
109
Adaptively segment objects from background
106
Advance stage to next well
116
N
Does the field contain objects?
107
Y
Acquire field for other markers (color #2-4)
108
110
Locate one object
111
Does the object meet the valid cell criteria?
N
Y
112
Measure cell features and store image and location
N
Y
Need to find more cells in current well?
115
N
Done with current plate?
N
Any unprocessed objects in the current field?
Y
Y
114
113
117
118
119
120
Interactive Data Review Mode
Review Images of Cells
Review Data on Cell-by-Cell Basis
Review Data on Well-by-Well Basis
Generate Report on plate
Symbol key:
Automatic
Conditional
Interactive

Figure 10

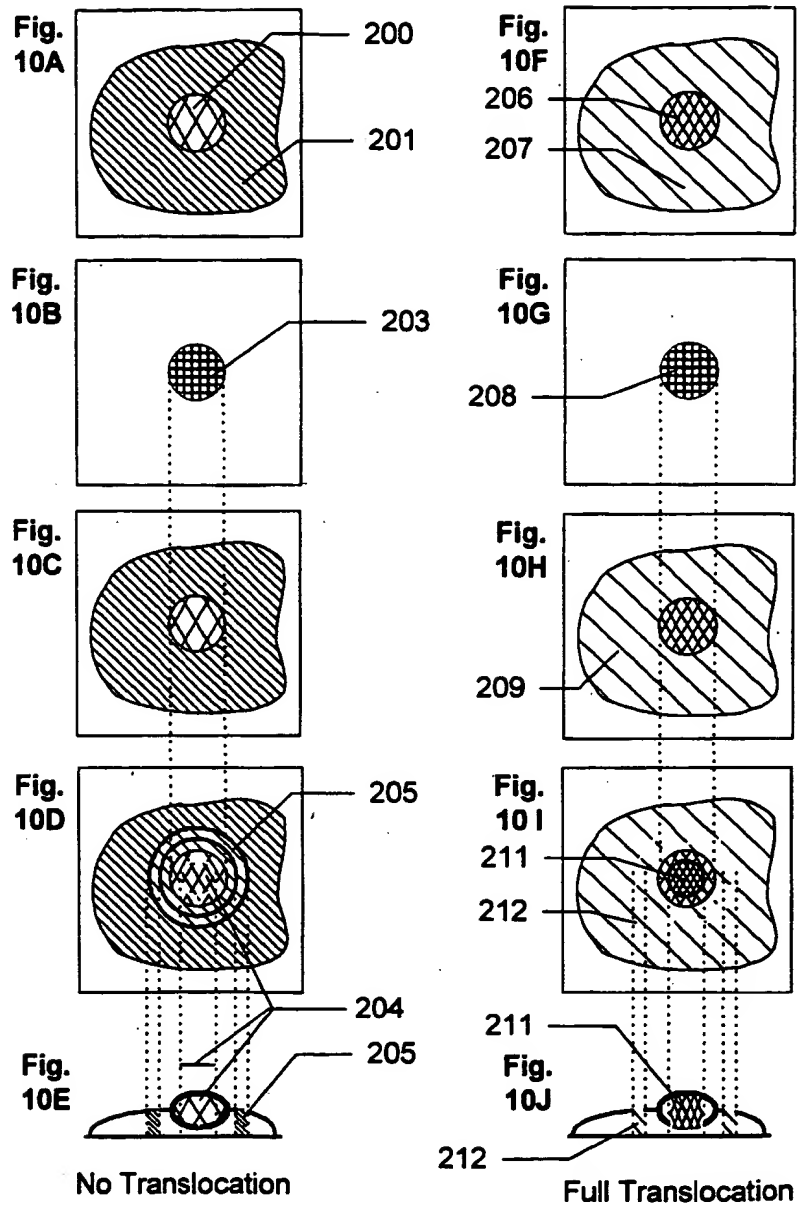


Fig. 10A
200
201
Fig. 10B
203
Fig. 10C
Fig. 10D
205
204
205
Fig. 10E
No Translocation
Fig. 10F
206
207
Fig. 10G
208
Fig. 10H
209
Fig. 10 I
211
212
211
Fig. 10J
212
Full Translocation

Figure 11

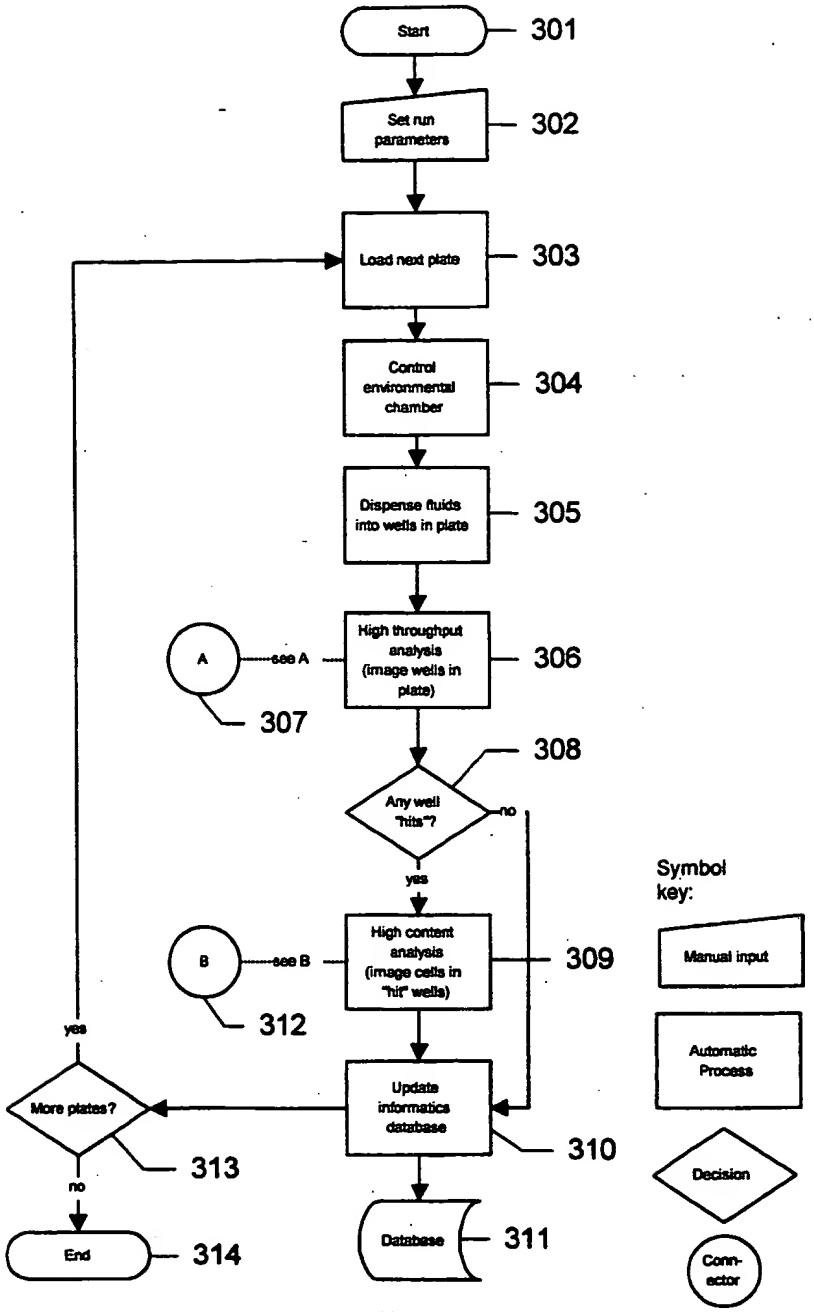

Start
301
Set run parameters
302
Load next plate
303
Control environmental chamber
304
Dispense fluids into wells in plate
305
High throughput analysis (image wells in plate)
306
A
see A
307
308
Any well "hits"?
no
yes
High content analysis (image cells in "hit" wells)
309
B
see B
312
Update informatics database
310
More plates?
yes
313
no
End
314
Database
311
Symbol key:
Manual input
Automatic Process
Decision
Conn-ector

Figure 12

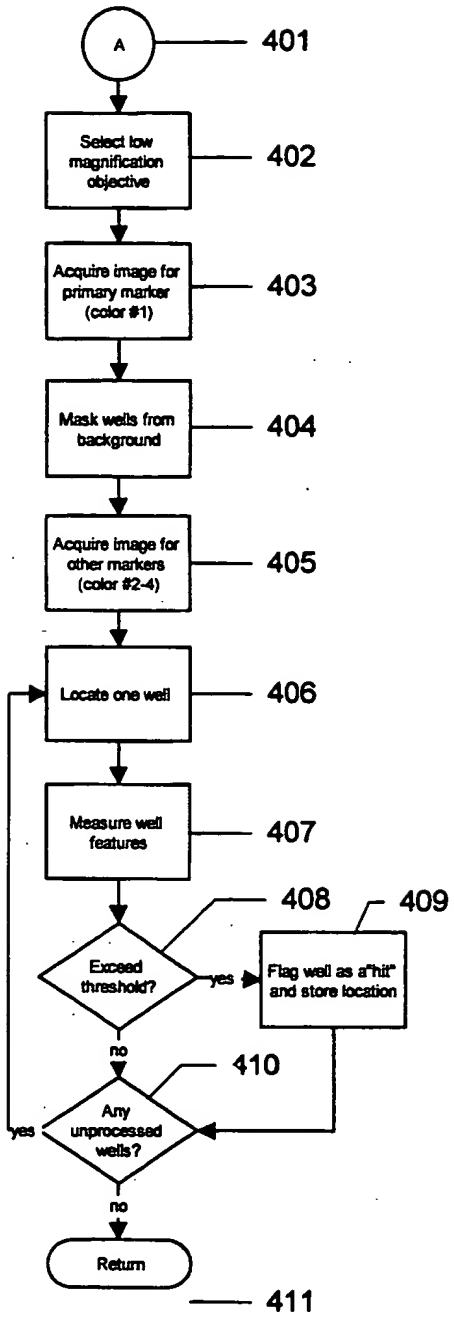

A
401
Select low magnification objective
402
Acquire image for primary marker (color #1)
403
Mask wells from background
404
Acquire image for other markers (color #2-4)
405
Locate one well
406
Measure well features
407
408
409
Exceed threshold?
yes
Flag well as a"hit" and store location
no
410
Any unprocessed wells?
yes
no
Return
411

Figure 13

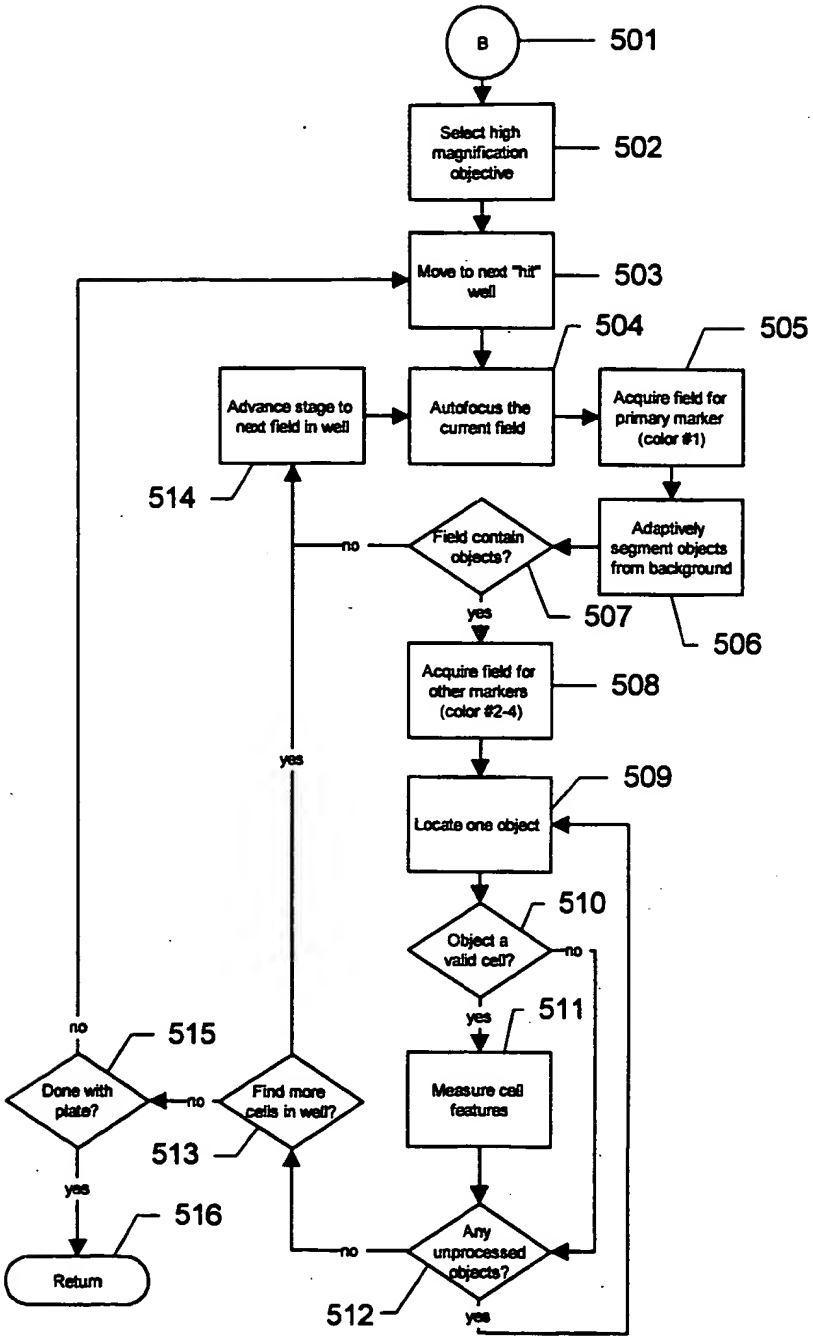

B
501
Select high magnification objective
502
Move to next "hit" well
503
504
505
Advance stage to next field in well
Autofocus the current field
Acquire field for primary marker (color #1)
514
Field contain objects?
no
yes
507
Adaptively segment objects from background
506
Acquire field for other markers (color #2-4)
508
yes
509
Locate one object
510
Object a valid cell?
no
yes
511
Measure cell features
no
515
Done with plate?
no
Find more cells in well?
513
yes
516
Return
Any unprocessed objects?
no
512
yes

Figure 14

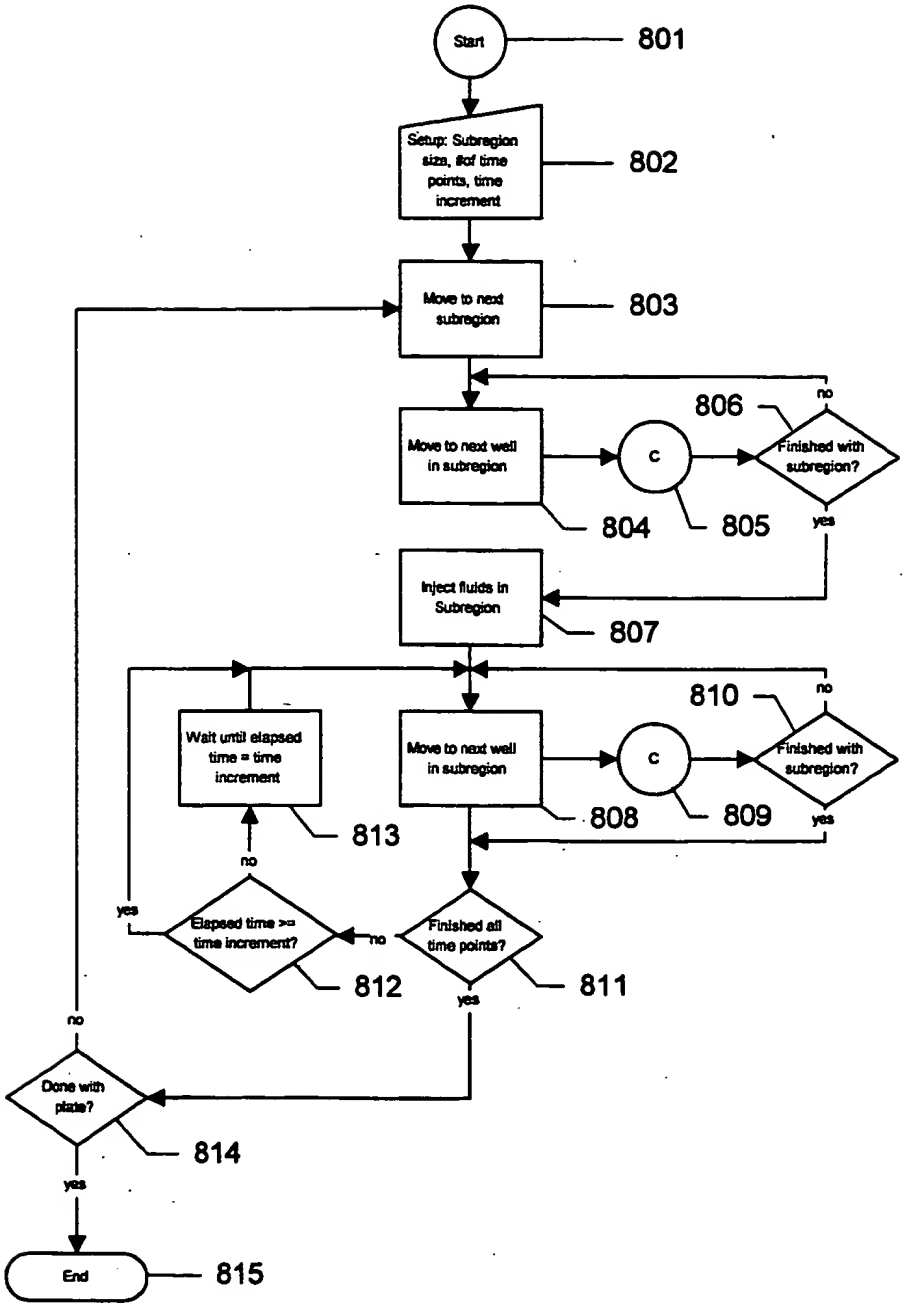

Start
801
Setup: Subregion size, #of time points, time increment
802
Move to next subregion
803
Move to next well in subregion
804
C
805
Finished with subregion?
806
no
yes
Inject fluids in Subregion
807
Move to next well in subregion
808
C
809
Finished with subregion?
810
no
yes
Finished all time points?
811
yes
no
Elapsed time >= time increment?
812
yes
no
Wait until elapsed time = time increment
813
no
Done with plate?
814
yes
no
End
815

Figure 15

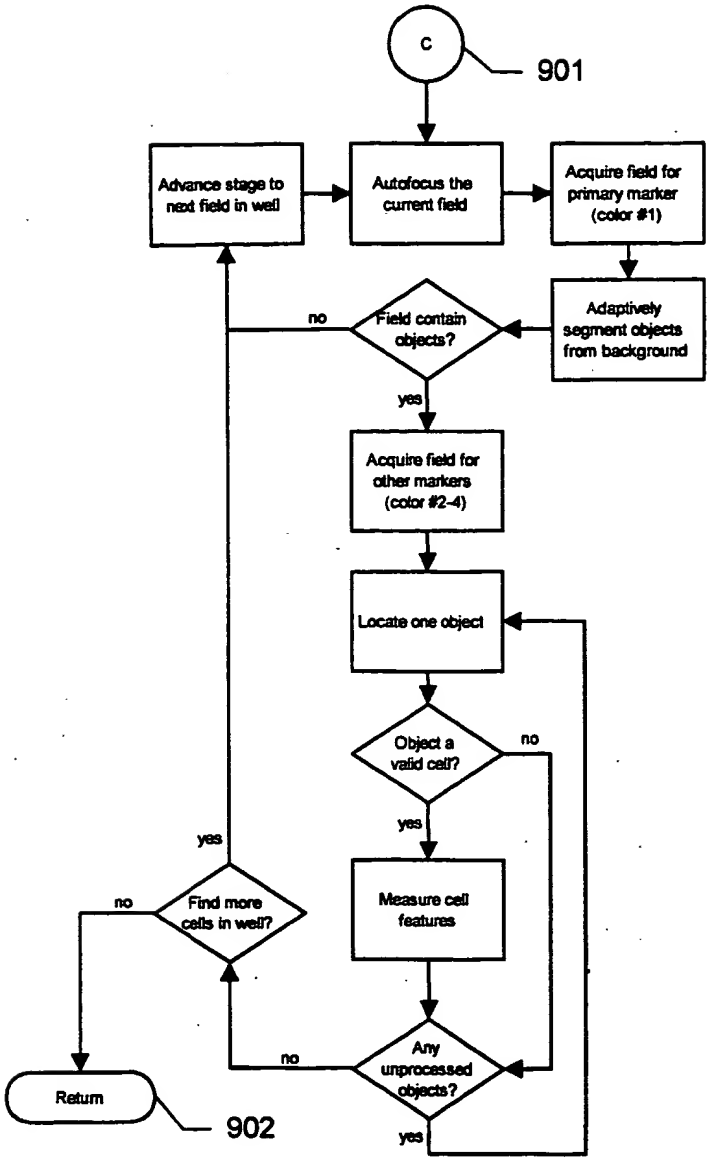

C
901
Advance stage to next field in well
Autofocus the current field
Acquire field for primary marker (color #1)
Adaptively segment objects from background
Field contain objects?
no
yes
Acquire field for other markers (color #2-4)
Locate one object
Object a valid cell?
no
yes
Measure cell features
Any unprocessed objects?
no
yes
Find more cells in well?
yes
no
Return
902

Figure 16

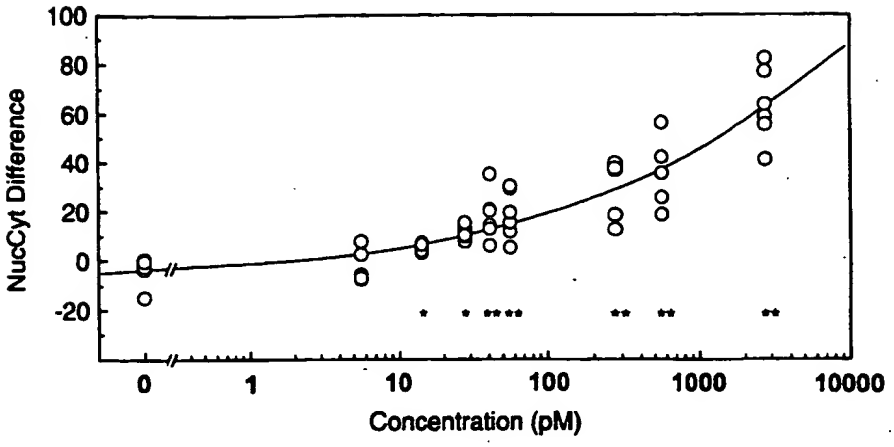

100
80
60
40
20
0
-20
NucCyt Difference
0
1
10
100
1000
10000
Concentration (pM)

Figure 17

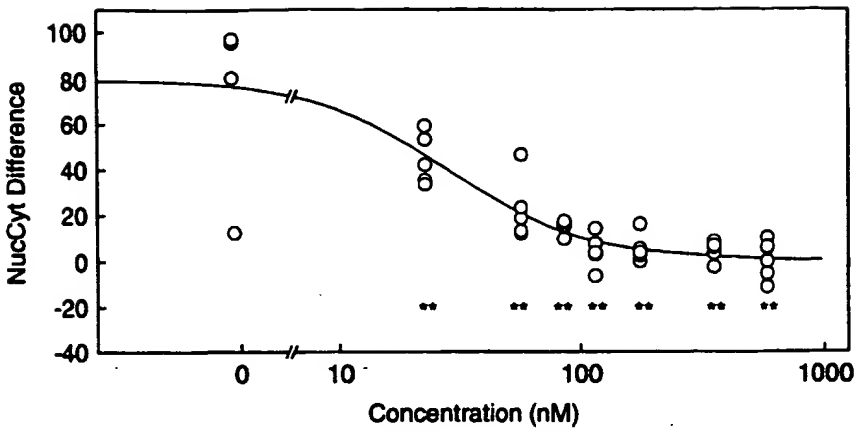

NucCyt Difference
100
80
60
40
20
0
-20
-40
0
10
100
1000
Concentration (nM)

Figure 18

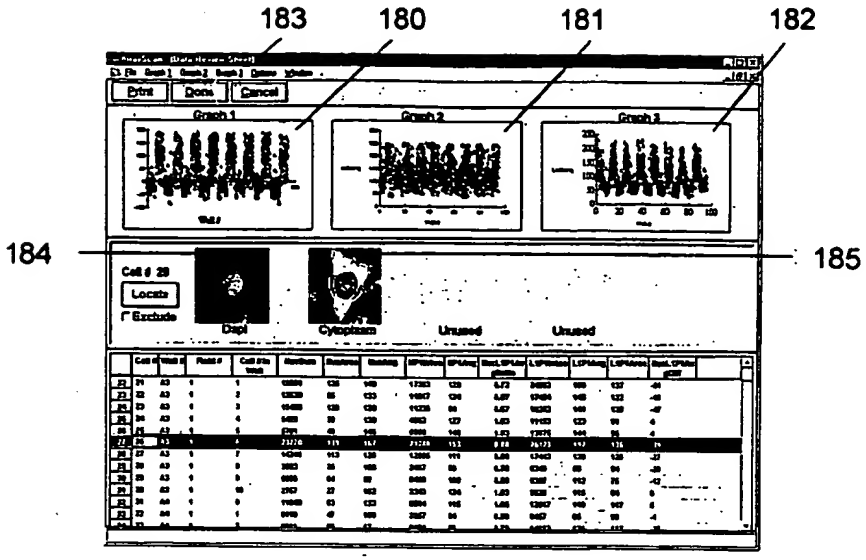

183
180
181
182
184
185
Print
Done
Cancel
Graph 1
Graph 2
Graph 3
Cell # 29
Locate
Exclude
Displ
Cytoplasm
Unused
Unused

Figure 19

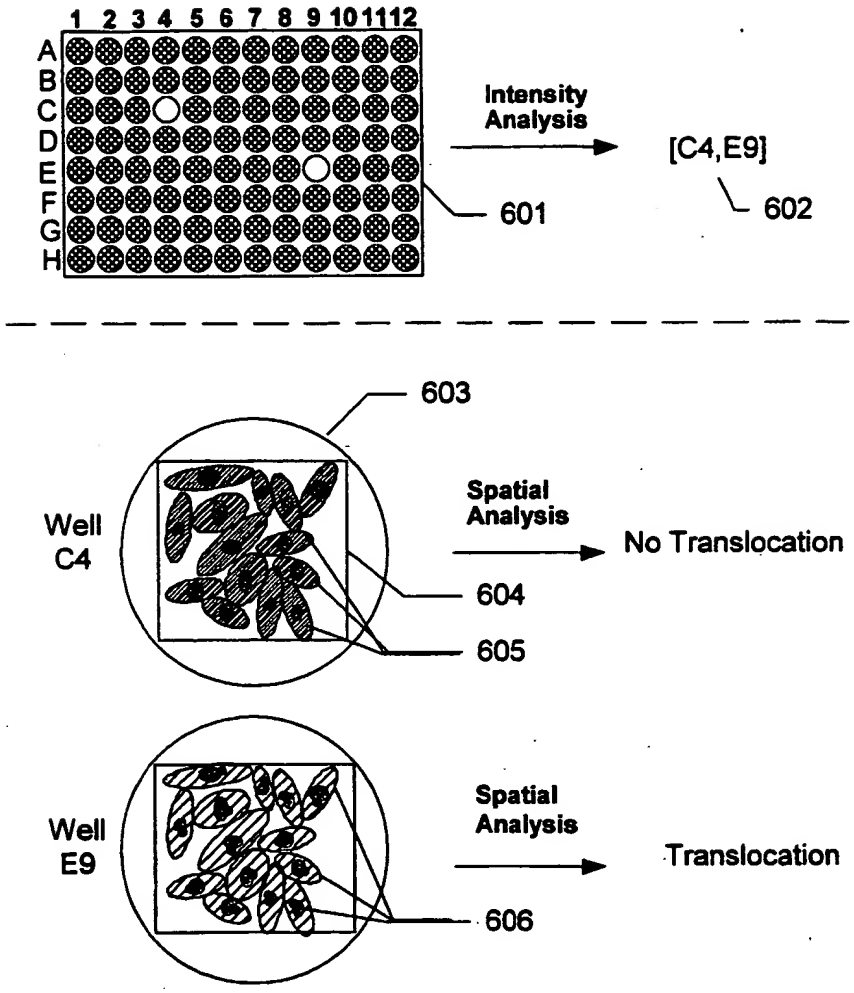

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Intensity Analysis
[C4,E9]
601
602
603
Well C4
Spatial Analysis
No Translocation
604
605
Well E9
Spatial Analysis
Translocation
606

Figure 20

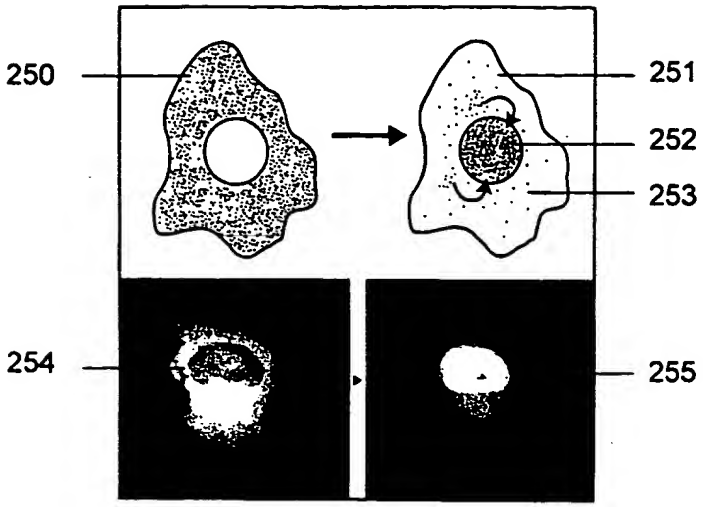


Fig. 20A Fig. 20B

Figure 21

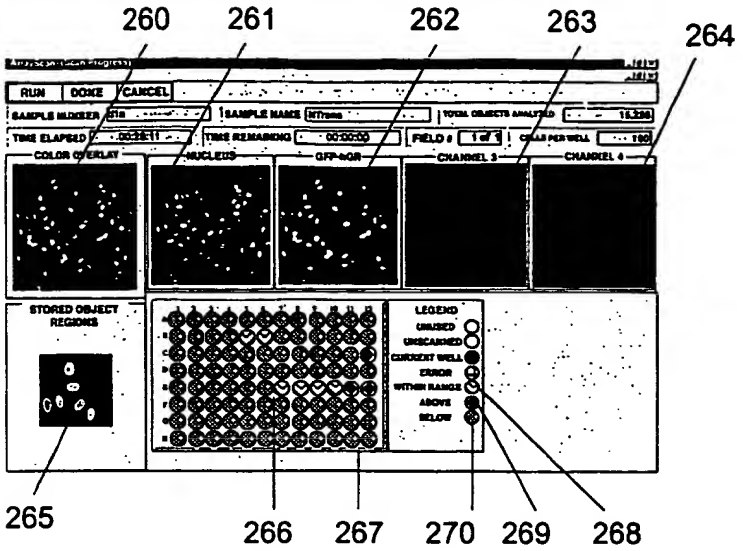

260
261
262
263
264
RUN
DONE
CANCEL
COLOR OVERLAY
NUCLEUS
GFP-wGR
CHANNEL 3
CHANNEL 4
STORED OBJECT REGIONS
LEGEND
UNUSED
UNSCANNED
CURRENT WELL
ERROR
WITHIN RANGE
ABOVE
BELOW
265
266
267
270
269
268

Figure 22

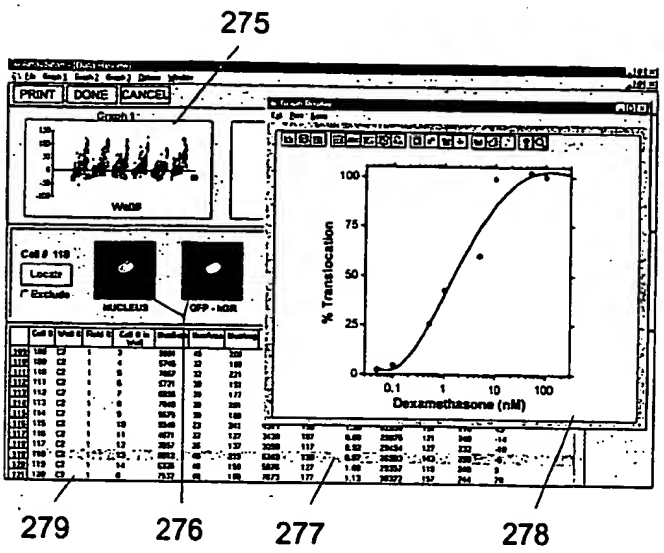

275
PRINT
DONE
CANCEL
Wells
NUCLEUS
% Translocation
Dexamethasone (nM)
279
276
277
278

Figure 23

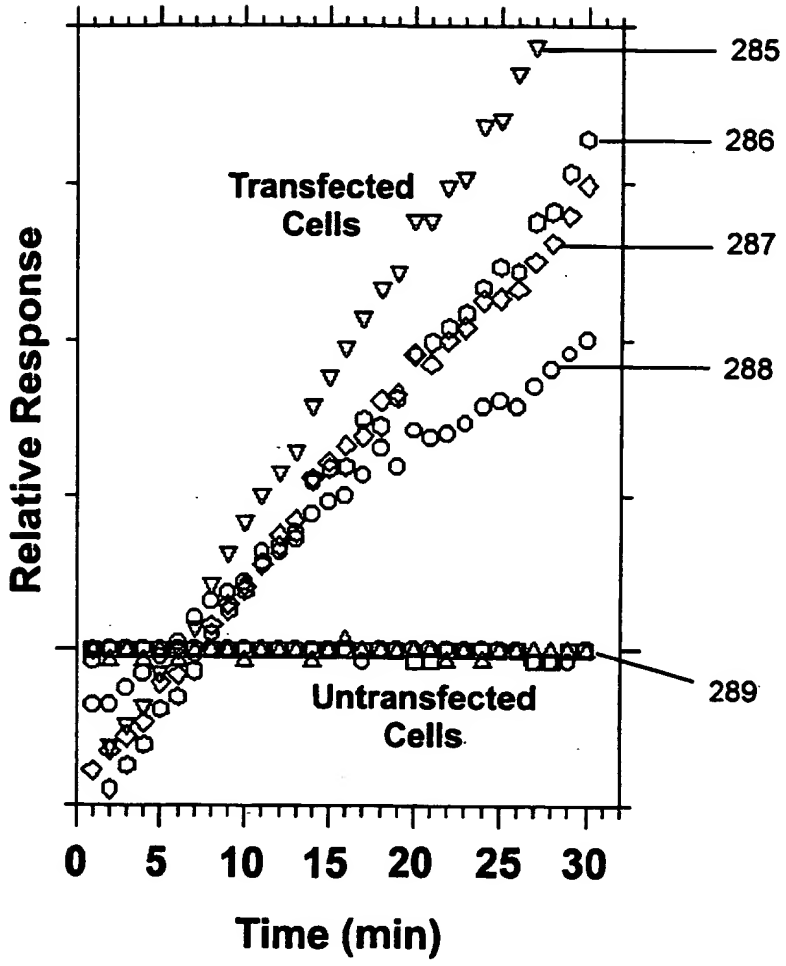

285
286
287
288
289
Transfected Cells
Untransfected Cells
Relative Response
0
5
10
15
20
25
30
Time (min)

Figure 24

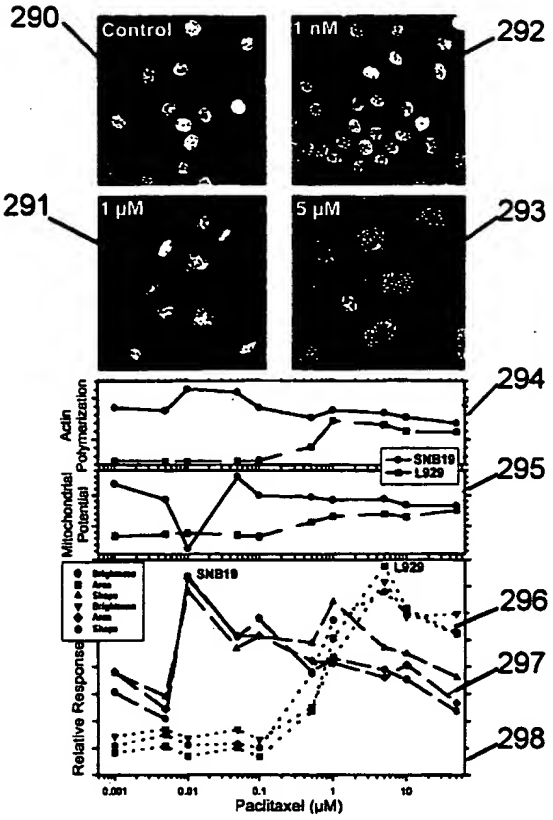

290
Control
1 nM
292
291
1 μM
5 μM
293
294
Actin Polymerization
SNB19
L929
295
Mitochondrial Potential
SNB19
L929
296
297
298
Relative Response
0.001
0.01
0.1
1
10
Paclitaxel (μM)

Figure 25

FIGURE 25

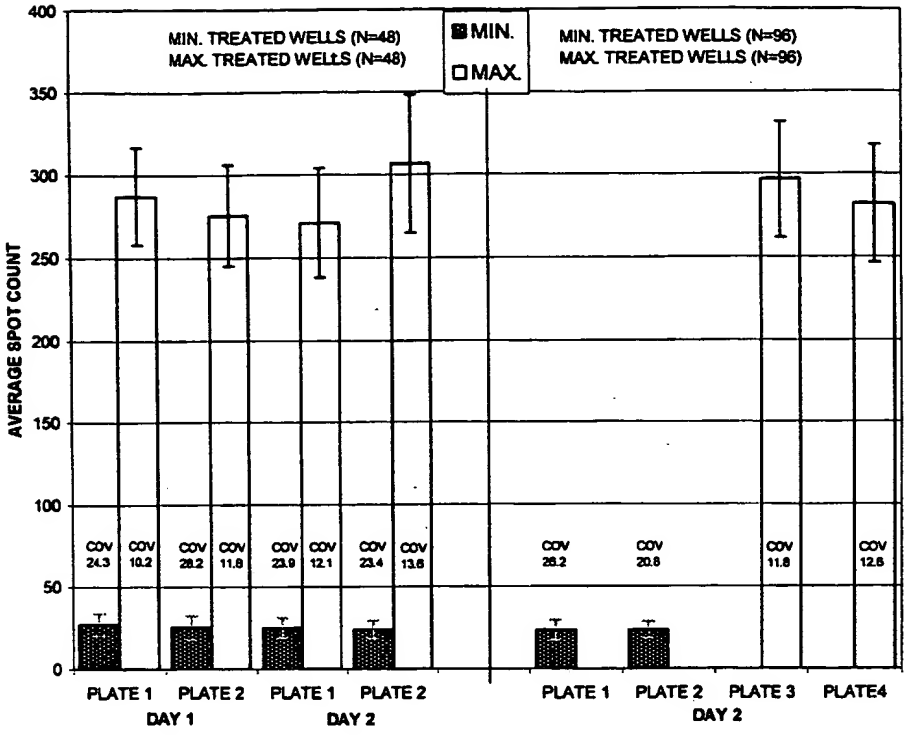


Figure 26

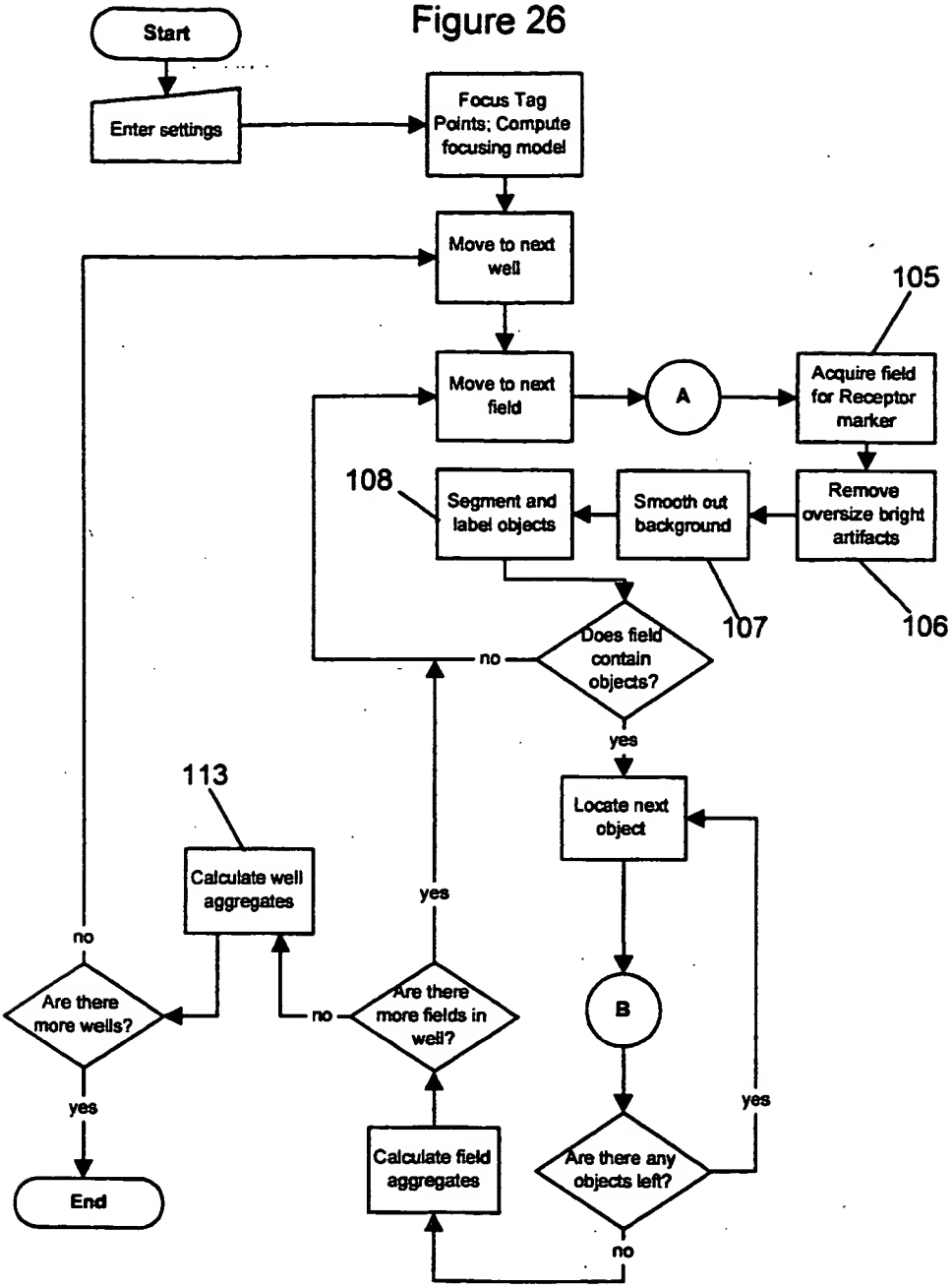

Start
Enter settings
Focus Tag Points; Compute focusing model
Move to next well
Move to next field
A
105
Acquire field for Receptor marker
Remove oversize bright artifacts
106
Smooth out background
107
108
Segment and label objects
Does field contain objects?
no
yes
Locate next object
B
Are there any objects left?
yes
no
Calculate field aggregates
Are there more fields in well?
yes
no
113
Calculate well aggregates
Are there more wells?
no
yes
End

Figure 27

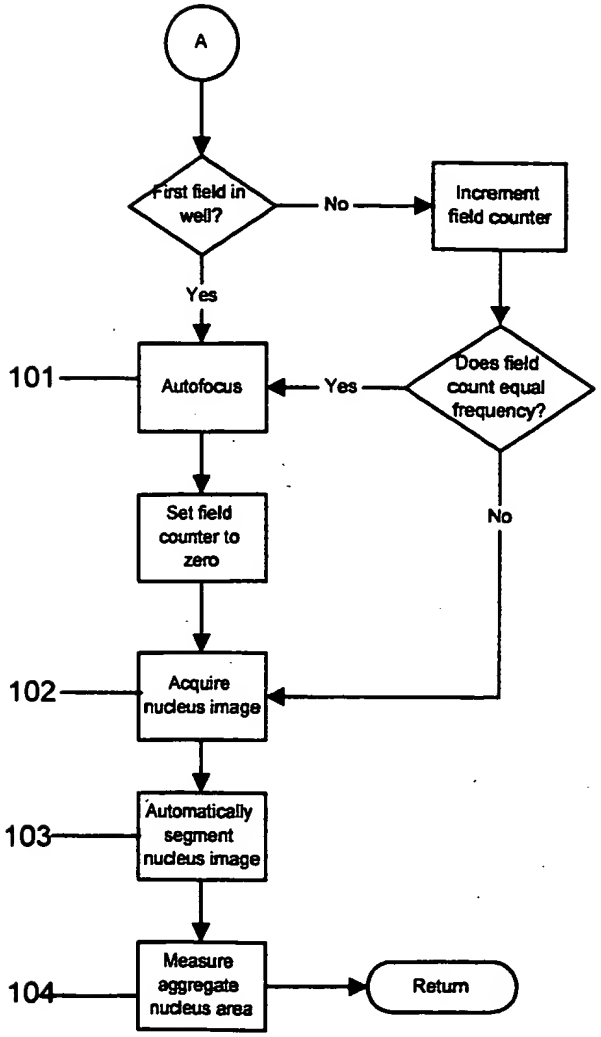

A
First field in well?
No
Increment field counter
Yes
Does field count equal frequency?
Yes
No
101
Autofocus
Set field counter to zero
102
Acquire nucleus image
103
Automatically segment nucleus image
104
Measure aggregate nucleus area
Return

Figure 28

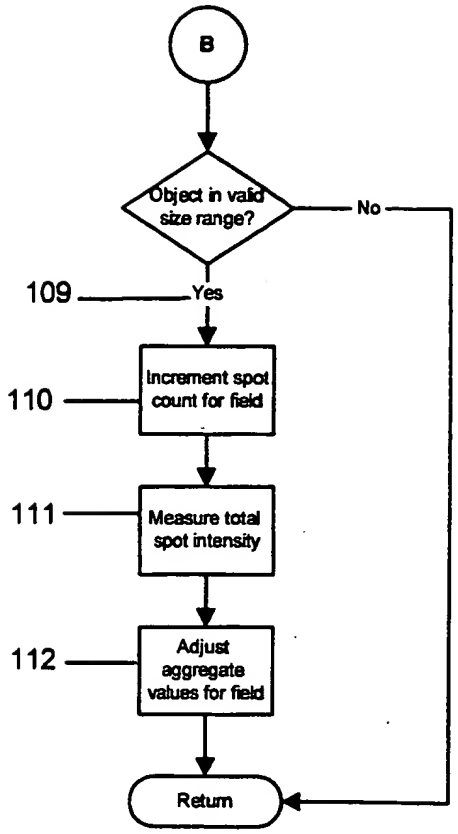

B
Object in valid size range?
No
109
Yes
Increment spot count for field
110
Measure total spot intensity
111
Adjust aggregate values for field
112
Return

Figure 29

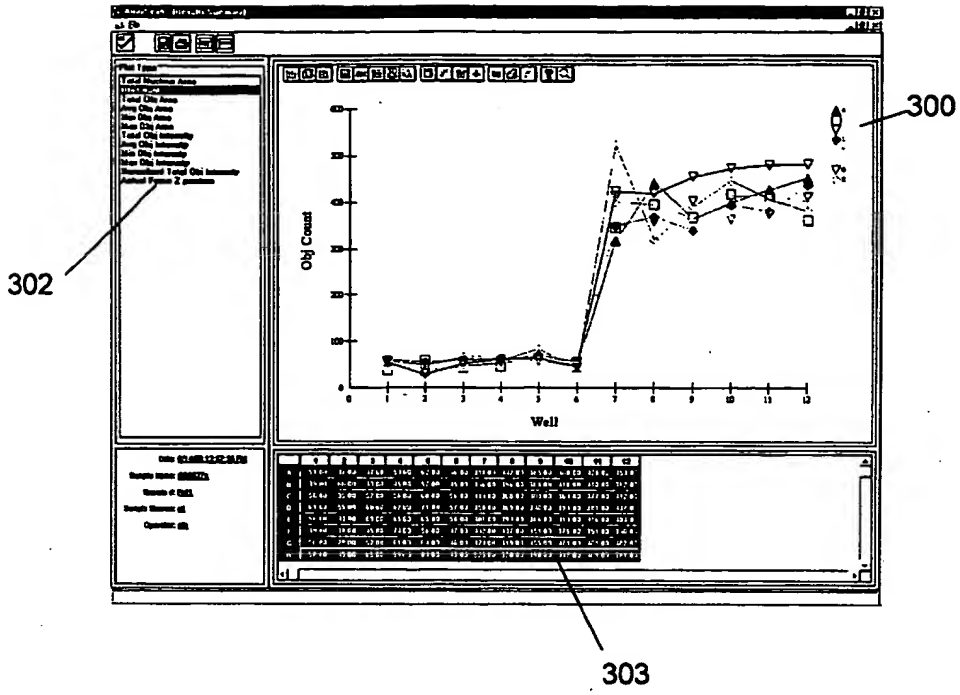


Figure 30

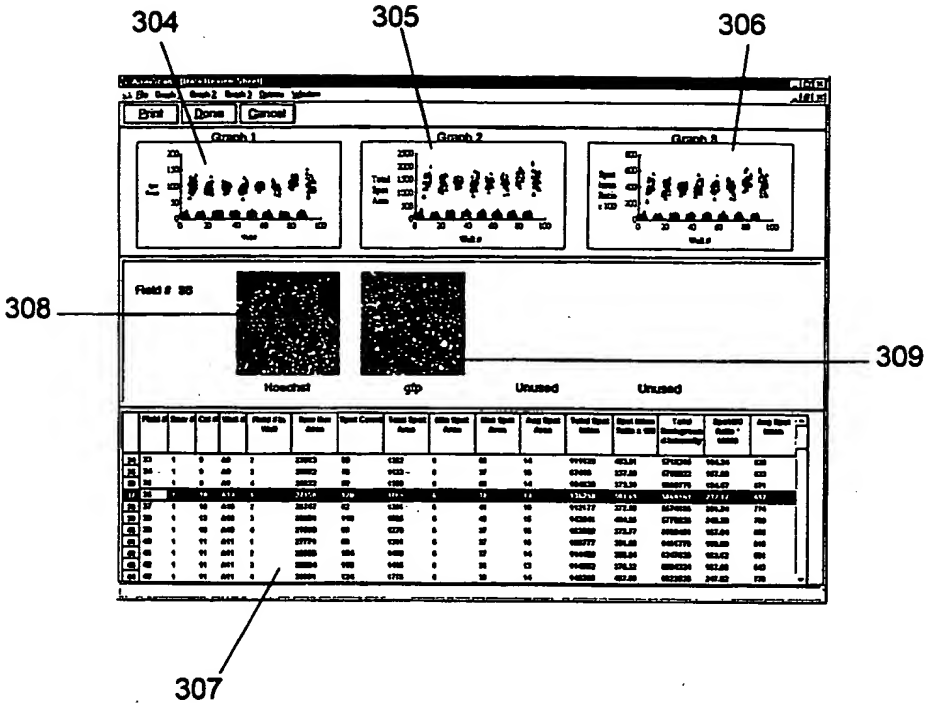

304
305
306
Print
Done
Cancel
Graph 1
Graph 2
Graph 3
308
309
Hoechst
gfp
Unused
Unused
307

Figure 31A

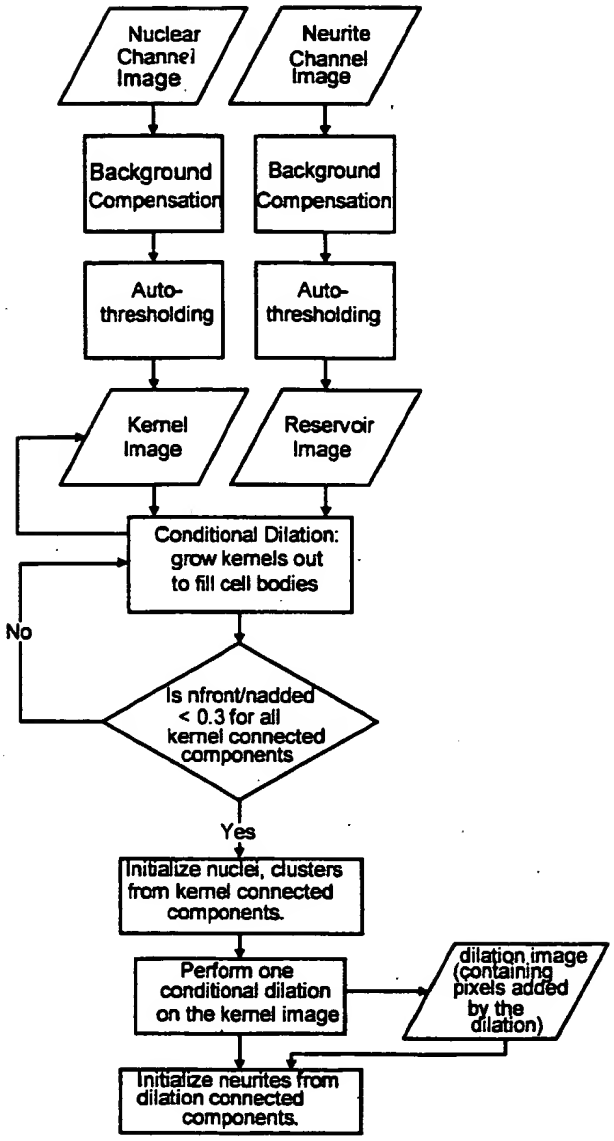

Nuclear Channel Image
Neurite Channel Image
Background Compensation
Background Compensation
Auto-thresholding
Auto-thresholding
Kernel Image
Reservoir Image
Conditional Dilation: grow kernels out to fill cell bodies
No
Is nfront/nadded < 0.3 for all kernel connected components
Yes
Initialize nuclei, clusters from kernel connected components.
Perform one conditional dilation on the kernel image
dilation image (containing pixels added by the dilation)
Initialize neurites from dilation connected components.

Figure 31B

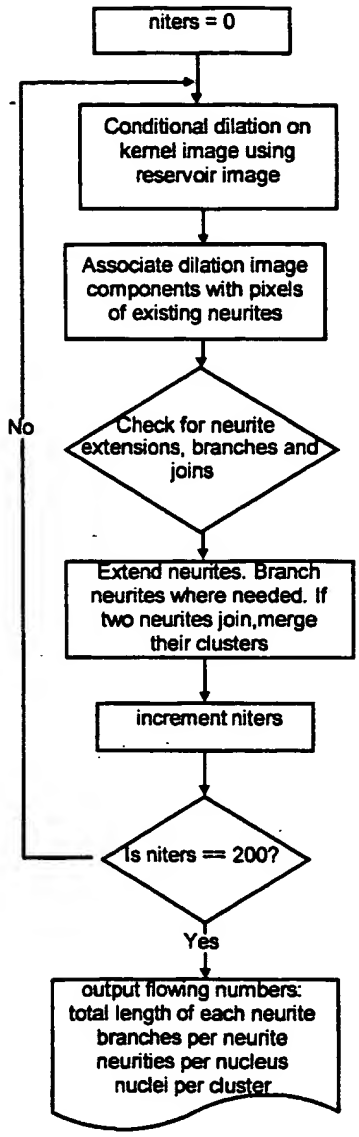

niters = 0
Conditional dilation on kernel image using reservoir image
Associate dilation image components with pixels of existing neurites
Check for neurite extensions, branches and joins
No
Extend neurites. Branch neurites where needed. If two neurites join,merge their clusters
increment niters
Is niters == 200?
Yes
output flowing numbers:
total length of each neurite
branches per neurite
neurities per nucleus
nuclei per cluster

Figure 32

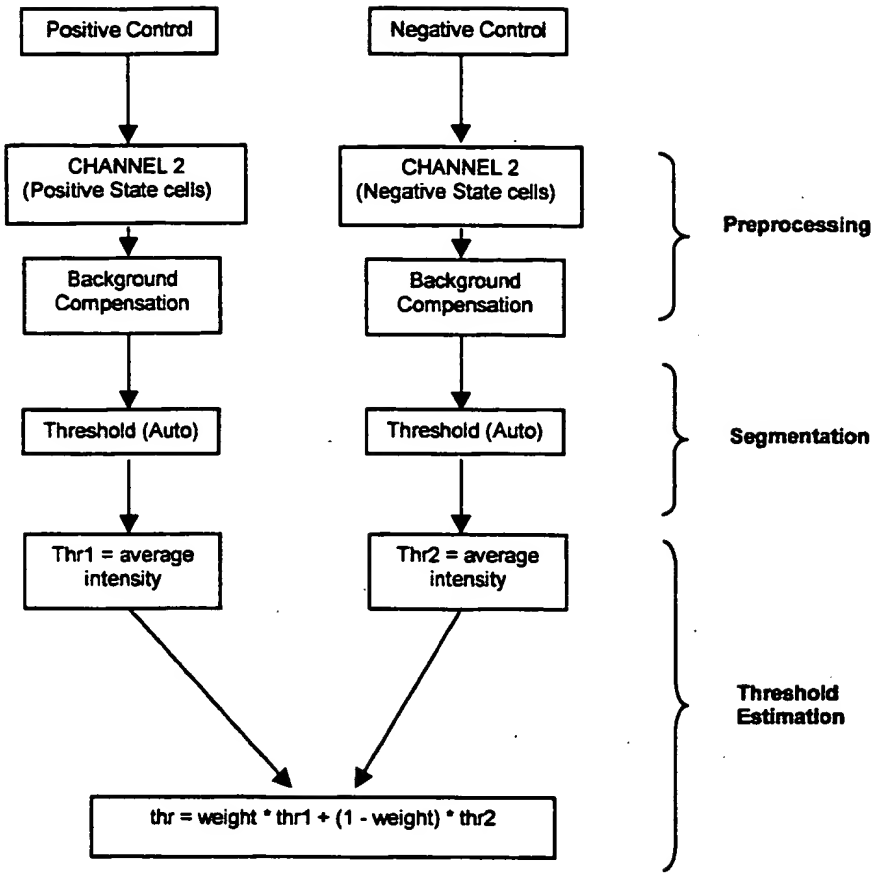

Positive Control
Negative Control
CHANNEL 2 (Positive State cells)
CHANNEL 2 (Negative State cells)
Background Compensation
Background Compensation
Threshold (Auto)
Threshold (Auto)
Thr1 = average intensity
Thr2 = average intensity
thr = weight * thr1 + (1 - weight) * thr2
Preprocessing
Segmentation
Threshold Estimation

Figure 33A

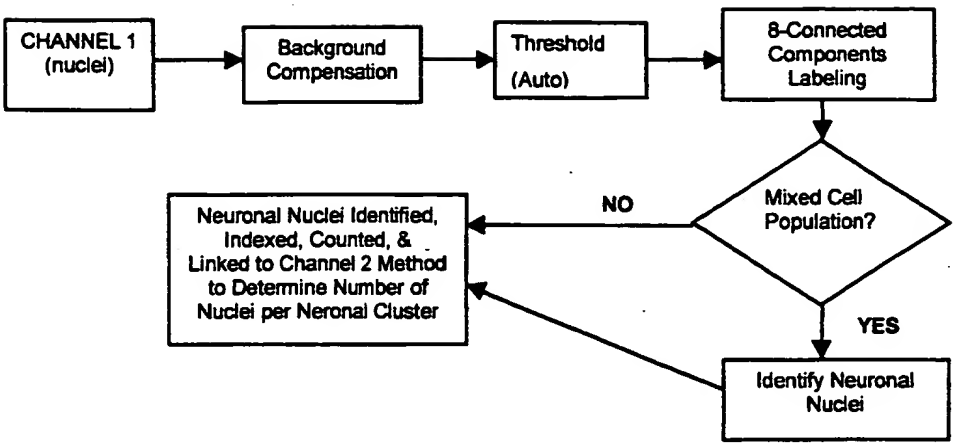

CHANNEL 1 (nuclei)
Background Compensation
Threshold (Auto)
8-Connected Components Labeling
Mixed Cell Population?
NO
YES
Neuronal Nuclei Identified, Indexed, Counted, & Linked to Channel 2 Method to Determine Number of Nuclei per Neronal Cluster
Identify Neuronal Nuclei

Figure 33B

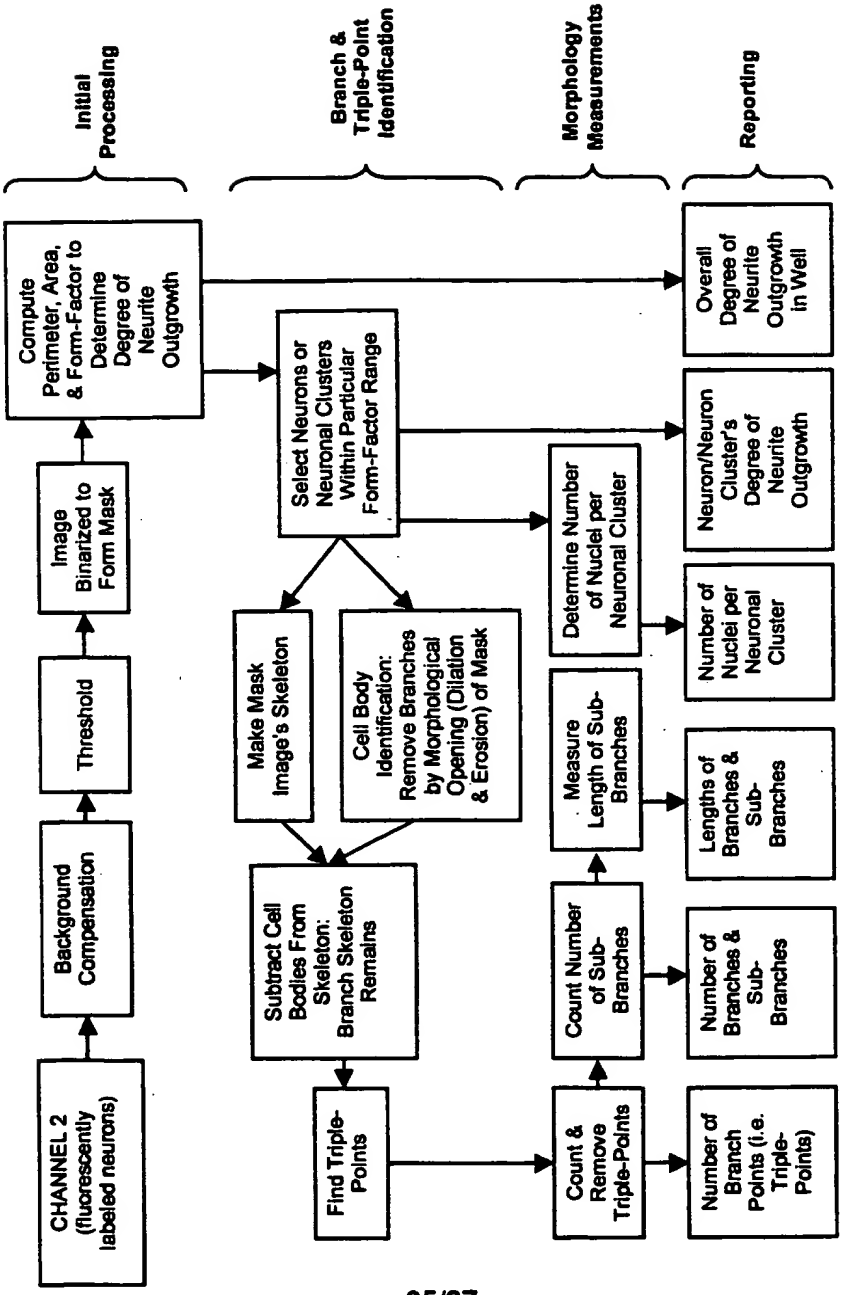

Initial Processing
Branch & Triple-Point Identification
Morphology Measurements
Reporting
CHANNEL 2 (fluorescently labeled neurons)
Background Compensation
Threshold
Image Binarized to Form Mask
Compute Perimeter, Area, & Form-Factor to Determine Degree of Neurite Outgrowth
Overall Degree of Neurite Outgrowth in Well
Select Neurons or Neuronal Clusters Within Particular Form-Factor Range
Neuron/Neuron Cluster's Degree of Neurite Outgrowth
Make Mask Image's Skeleton
Cell Body Identification: Remove Branches by Morphological Opening (Dilation & Erosion) of Mask
Determine Number of Nuclei per Neuronal Cluster
Number of Nuclei per Neuronal Cluster
Subtract Cell Bodies From Skeleton: Branch Skeleton Remains
Measure Length of Sub-Branches
Lengths of Branches & Sub-Branches
Find Triple-Points
Count Number of Sub-Branches
Number of Branches & Sub-Branches
Count & Remove Triple-Points
Number of Branch Points (i.e. Triple-Points)

Figure 34

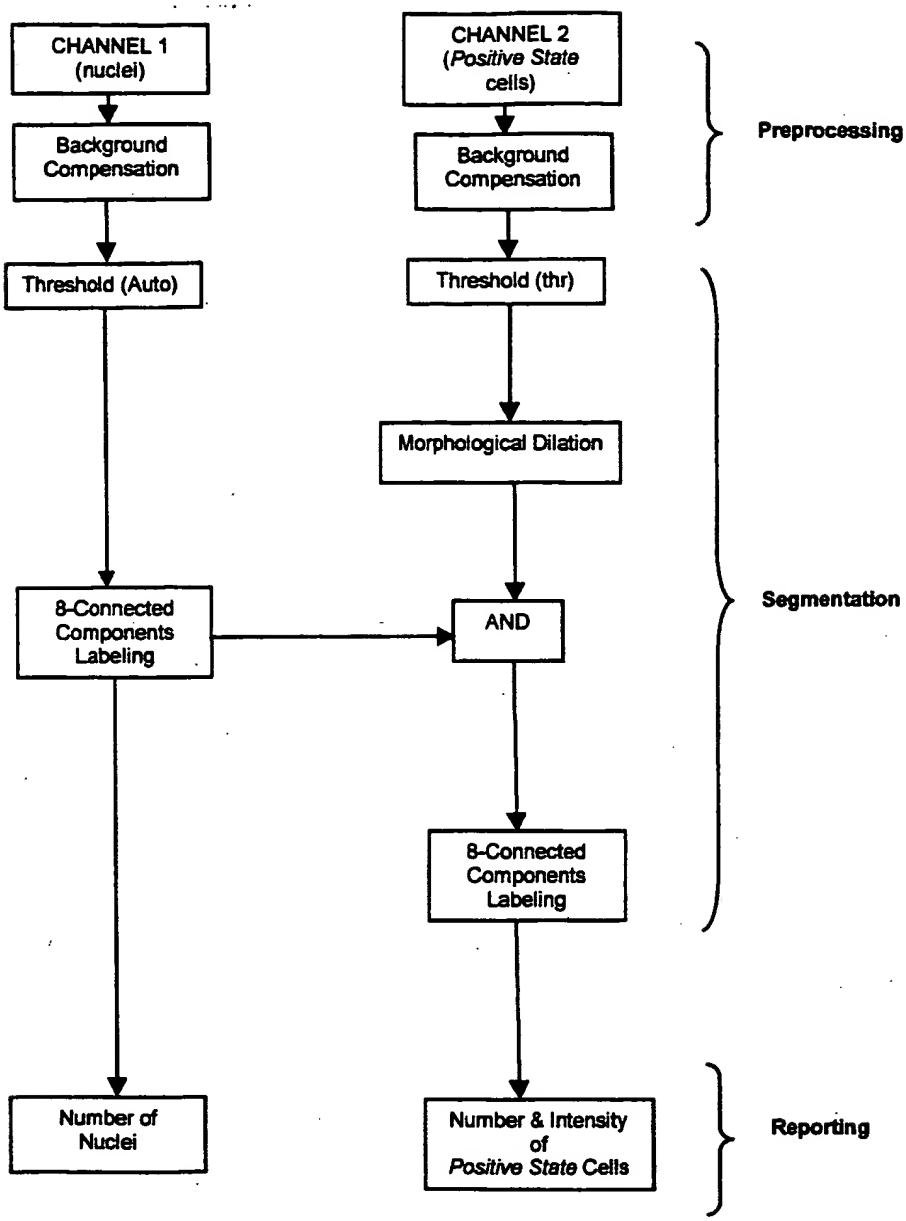
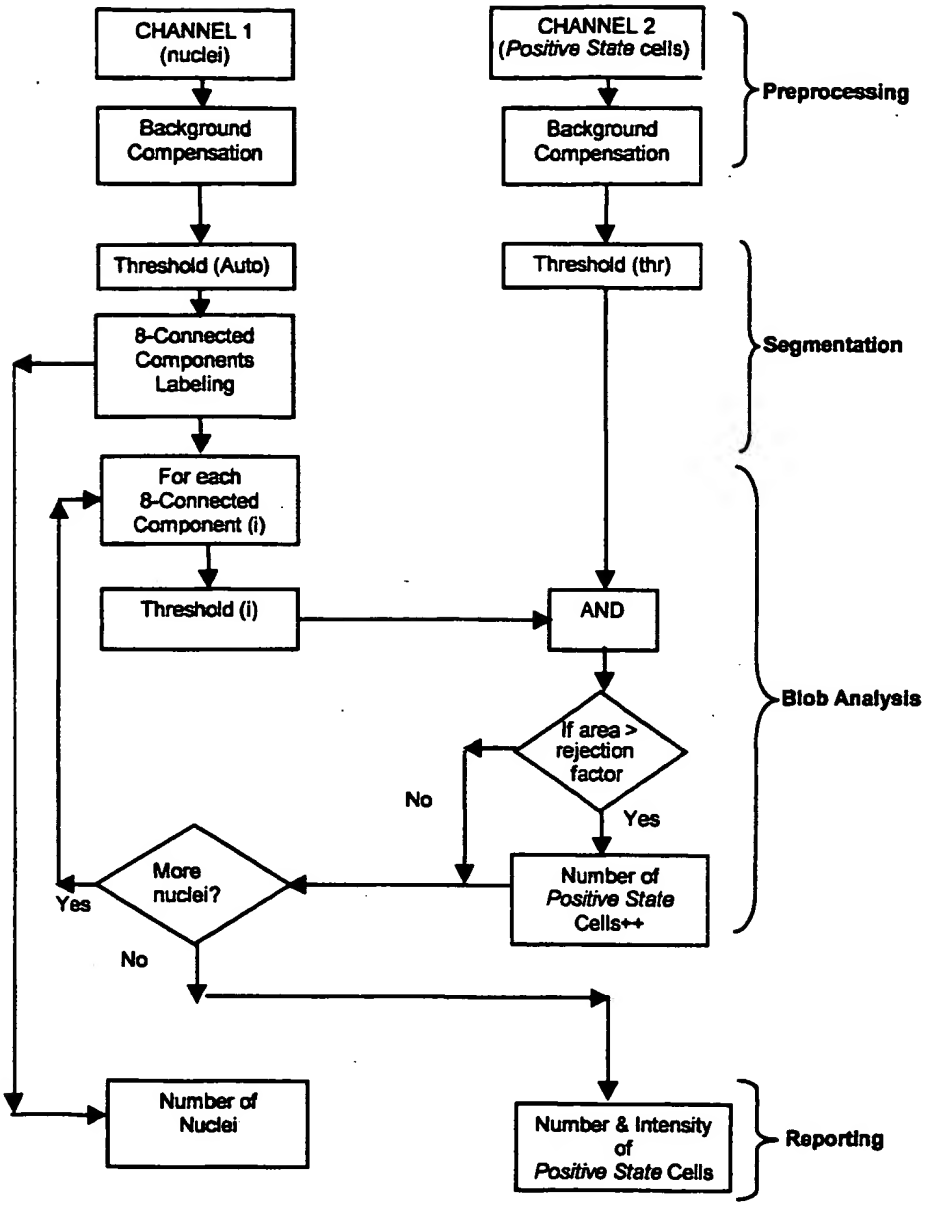

CHANNEL 1 (nuclei)
CHANNEL 2 (*Positive State* cells)
Background Compensation
Background Compensation
Preprocessing
Threshold (Auto)
Threshold (thr)
Morphological Dilation
8-Connected Components Labeling
AND
Segmentation
8-Connected Components Labeling
Number of Nuclei
Number & Intensity of *Positive State* Cells
Reporting

Figure 35


CHANNEL 1 (nuclei)
CHANNEL 2 (Positive State cells)
Preprocessing
Background Compensation
Background Compensation
Threshold (Auto)
Threshold (thr)
Segmentation
8-Connected Components Labeling
For each 8-Connected Component (i)
Threshold (i)
AND
Blob Analysis
If area > rejection factor
No
Yes
More nuclei?
Yes
No
Number of Positive State Cells++
Number of Nuclei
Number & Intensity of Positive State Cells
Reporting